Universidade de Aveiro Departamento de Química
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Armando da Costa Duarte, Professor Catedrático no Departamento de Química da Universidade de Aveiro, Doutora Ana Gomes, Professora Auxiliar na Escola Superior de Biotecnologia da Universidade Católica Portuguesa e da Doutora Ana Cristina Freitas, Professora Auxiliar no Instituto Superior de Estudos Interculturais e Transdisciplinares de Viseu do Instituto Piaget.

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Dedico este trabalho à minha família pelo incansável apoio.

o júri

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palavras-chave

Algas comestíveis, cogumelos comestíveis, métodos de extração sustentáveis e compatíveis com alimentos, caracterização química, propriedades biológicas, novos alimentos funcionais

resumo

Recursos naturais, como algas e cogumelos podem ser usados para a obtenção de novos produtos, oferecendo uma forma alternativa e sustentável de originar novos alimentos ou ingredientes funcionais com propriedades biológicas que podem ajudar a desenvolver novas estratégias baseadas na saúde preventiva. A principal força motriz desta tese foi encontrar em algas e cogumelos, extratos com compostos bioativos naturais que possam ser usados no desenvolvimento de novos alimentos funcionais. Com este objectivo, foi efectuada uma abordagem integrada e vários passos foram seguidos numa ordem cronológica. Em primeiro lugar, selecionou-se um conjunto de espécies de algas marinhas assim como um conjunto de espécies de cogumelos comestíveis que foram caracterizados quanto à sua composição química e compostos bioativos potenciais. Tendo sido realizada a sua caracterização, surgiu a necessidade de se aplicar metodologias de extracção adequadas, rentáveis e amigas do ambiente, capazes de extrair ingredientes naturais biologicamente ativos de interesse tanto de algas como de cogumelos. Este objetivo nesta fase levou à segunda etapa do trabalho experimental que envolveu o estudo de várias técnicas de extração (extração com água quente, extração assistida por enzimas e por ultra-sons e extracção sob alta pressão hidrostática), a fim de se caracterizar plenamente o potencial das diferentes fontes naturais, introduzindo em simultâneo diferentes seletividades e eficiências, preservando o máximo de bioatividade. A etapa seguinte desta pesquisa integrada esteve relacionada com a caracterização química mais aprofundada de compostos bioativos presentes nos quatro extratos enzimáticos selecionados de algas (extrato *S. muticum* obtido com Alcalase e extrato *O. pinnatifida* obtido com Viscozyme) e de cogumelos (extratos de *Ph. nameko* obtidos com Celulase e com Flavourzyme) onde as propriedades biológicas alvo foram confirmadas ou verificadas como as mais promissoras. Os extratos selecionados com potenciais propriedades biológicas, após a caracterização química foram avaliados no que respeita à sua estabilidade *in vitro* para confirmar e consolidar o seu potencial biológico e ser mais explorado na perspectiva do alimento funcional. A última etapa deste trabalho envolveu o desenvolvimento de um novo alimento funcional incorporando-se os dois extratos mais promissores e validados previamente (extrato *O. pinnatifida* obtido com Viscozyme e extrato *Ph. nameko* obtido com Flavourzyme) num creme lácteo para barrar, procedendo-se à avaliação do seu potencial biológico e tecnológico. Um creme lácteo de barrar funcional, combinando requeijão e iogurte tipo grego com incorporação dos extratos selecionados foi formulado e explorado com sucesso. O desenvolvimento de alimentos funcionais, ou mesmo de nutracêuticos, a partir de extratos de algas e de cogumelos comestíveis é viável podendo-se estender o estudo da incorporação destes extratos em outros tipos de alimentos como bebidas ou sorvete.

keywords

Edible seaweeds, edible mushrooms, sustainable extraction methods for food applications, chemical characterization, biological properties, new functional foods

abstract

Natural resources such as seaweed and mushrooms can be used to obtain new products, providing an alternative and sustainable manner to provide new functional foods or ingredients with biological properties that may help to develop new strategies based on preventive health. The main driving force of this thesis was to find in seaweeds and mushrooms, extracts with natural bioactive compounds to be used in the development of new functional foods. In order to do so, an integrated approach was established and the various steps were followed chronologically. Firstly, a set of edible seaweeds species and a set of edible mushrooms species were characterised for their proximate composition and potential bioactive compounds. Once the characterization was achieved the need for suitable, fast, cost-effective and environmentally friendly extraction methodologies capable of extracting the biologically active natural ingredients of interest from both the seaweeds and mushrooms led to the second stage. This stage involved the study of several extraction techniques (hot water extraction; enzyme- and ultrasound-assisted extraction and high hydrostatic pressure) in order to fully characterize the potential of the different natural sources, introducing different extraction selectivity and efficiency while aiming at maximum preservation of bioactivity. The next stage in this integrated research was related with the deeper chemical characterization of the bioactive components present in the four enzymatic selected extracts from seaweeds (*S. muticum* extract obtained with Alcalase and *O. pinnatifida* extract obtained with Viscozyme) and mushrooms (*Ph. nameko* extracts obtained with Cellulase and with Flavourzyme) where target biological properties were confirmed or found to be more promising. The selected extracts with potential biological properties, following the chemical characterization went through the evaluation of *in vitro* stability to confirm and consolidate its biological potential to be further explored within the functional food perspective. The last stage of this thesis involved the development of a new functional food by incorporating the two most promising and validated extracts (*O. pinnatifida* obtained with Viscozyme and *Ph. nameko* obtained with Flavourzyme) in a spreadable dairy cream with assessment of their biological and technological potential. A functional dairy spreadable dairy cream combining whey cheese and greek type yoghurt with incorporation of the selected extracts was successfully formulated and explored. The development of functional foods, or even nutraceuticals, from edible seaweed and mushroom extracts is feasible and could be extended studying the incorporation of these extracts in other types of food such as beverages or ice cream.

AA	Arachidonic acid
ABTS	Antioxidant capacity
ACE	Angiotensin-I converting enzyme
Alc	Alcalase
ANOVA	Analysis of variance
ARA	Arachidonic acids
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CE	Conventional extraction
Cell	Cellulase
CHD	Coronary heart diseases
CVD	Cardiovascular diseases
DHA	Docosahexaenoic
DPA	Docosapentaenoic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EA	Enzymatic extracts
EAE	Enzyme-assisted extraction
EPA	Eicosapentaenoic
FA	Fatty acids
FF	Functional foods
FFI	Functional food ingredients
FIP	Fungal immune-modulator proteins
Flav	Flavourzyme
FOS	Fructooligosaccharides
FTIR-ATR	Fourier Transform Infrared Spectroscopy with attenuated total reflectance
GIT	Gastrointestinal Tract
HHP	High hydrostatic pressure
HWE	Hot water extraction
ICP	Inductively coupled plasma
LOD	Limit of detection
MUFA	Monounsaturated fatty acid
NBT	Nitroblue tetrazolium
OES	Optical emission spectrometer
OPA	Orthophthalaldehyde
PBS	Phosphate Buffered Saline
PPAR-alpha	Peroxisome proliferator-activated receptor-alpha
PUFA	Polyunsaturated Fatty acid
RDIs	Recommended daily intakes
RIP	Ribosome inactivating proteins
RXR	Retinoid X receptor

SCFA	Short chain fatty acids
SD	Standard deviation
SFA	Saturated fatty acid
SHRs	Spontaneously hypertensive rats
SWE	Subcritical water extraction
TMCSI	Trimethylchlorosilane
UAE	Ultrasound-assisted extraction
UVB	Ultraviolet B
Visc	Viscozyme

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Chapter 1

Objectives and Thesis Outline

1.1 Objectives

The use of functional foods can provide numerous nutritional benefits to the consumer contributing to the maintenance of health and reduction of risk of certain diseases. Seaweeds and mushrooms represent a great source of new untapped ingredients that may be considered healthy, biologically active compounds to be exploited for food applications. Functional foods (FF), including those of dairy origin, are already widely consumed throughout the world for their health benefits and are therefore a potential vector to be considered for the incorporation of new functional ingredients derived from seaweeds and/ or mushrooms, which, in turn, will further enhance their nutritional value. The development of a FF involves a number of steps ranging from the identification of ingredients with beneficial properties to the validation of their efficacy and safety.

Based on the above rationale, the main objective of this thesis is the development of new FF by incorporation of extracts with bioactive compounds from seaweeds and/ or mushrooms, a field yet to be exploited, given the absence of literature studies in this domain, in particular FF with seaweeds' extracts. The scope of this thesis covers a wide range of scientific areas from chemistry, biochemistry to biotechnology and therefore the general objective can be divided into the following specific objectives:

i) Contribution to biotechnological research studies on seaweeds and mushrooms as potential sources of functional ingredients;

ii) Chemical characterization and nutritive evaluation of a selection of edible seaweeds from the Portuguese Coast;

iii) Chemical characterization and nutritive evaluation of a selection of edible and cultivated mushrooms;

iv) Comparison of different extraction methods and their impact on the chemical and biological properties of extracts from seaweeds and mushrooms;

v) Selection of the more promising extracts and their characterization in terms of chemical nature and biological properties;

vi) Development of new FF based on a dairy spreadable cream with incorporated extracts.

In order for these goals to be achieved, several major steps were performed for this PhD thesis:

1. Search and selection of edible seaweeds and mushrooms for the identification of functional ingredients;

2. Use of several extraction methods-based processes that are sustainable and food compatible;

3. Chemical characterization and evaluation of biological potential by evaluating antioxidant, antihypertensive, antidiabetic, and prebiotic properties of seaweeds' or mushrooms' extracts;

4. Selection of extracts with functional ingredients, followed by characterization and evaluation of stability and bioavailability *in vitro*.

5. Development of FF by incorporating previously validated extracts in food matrices and assessment of their biological and technological potential.

1.2. Thesis outline

The schematic representation of the developed experimental work is displayed in Figure 1.1. The work disclosed in this thesis was presented in the format of five posters at national and international meetings, two papers in conference proceedings, five papers published as well as two papers submitted in peer-reviewed international journals in the Science Citation Index (SCI®, Thomson ISI), one chapter in a Book and three papers in preparation. This thesis is composed of eleven chapters organized as follows:

- Chapter 1 provides the general and specific objectives of this thesis, as well as its organization in various chapters with reference to their associated contents;

- Chapter 2 gives an up to date introduction on the theoretical relevance of the various topics explored within this thesis summarizing the main scientific studies available on each topic. These include functional ingredients from seaweeds and mushrooms and their biological properties, food compatible and sustainable extraction methods and FF incorporating natural-derived products, extracts as wells as current perspectives on the subjects.

Chapter 2 is partially based on the following publications:

Freitas AC, Pereira L, Rodrigues D, Carvalho AC, Panteleitchouk T, Gomes AM, Duarte AC. **2015.** Chapter 42 - Marine functional foods. *In* Springer Handbook of Marine Biotechnology. Trends and Industrial Applications, Kim Se-Kwon (Ed). Springer. Hardback/Paper version: ISBN 978-3-642-53970-1; Ebook - ISBN 978-3-642-53971-8; 1560 p (pp. 969-994). Doi:10.1007/978-3-642-53971-8_42.

Rodrigues D, Rocha-Santos TAP, Freitas AC, Gomes AMP, Duarte AC. **2012**. Analytical strategies for characterization and validation of functional dairy foods. *Trends in Analytical Chemistry* 41, 27-45. *(IF (2012)= 6.351; Rank= 2/75; Q1; Area: Chemistry Analytical).*

Freitas AC, Rodrigues D, Rocha-Santos TAP, Gomes AMP, Duarte AC. **2012**. Marine Biotechnology advances towards applications in new functional foods. *Biotechnology Advances* 30, 1506-1515. *(IF (2012)= 9.559; Rank= 6/159; Q1; Area: Biotechnology & Applied Microbiology).*

- Chapter 3 presents the chemical composition and nutritive value of a selection of six edible seaweeds collected in Buarcos bay in Central West Coast of Portugal. The selection of species,

their elemental and proximate composition, fatty acids profile as well as FTIR-ATR and FT-Raman characterization is deeply presented and discussed.

Chapter 3 is partially based on the following publication:

Rodrigues D, Freitas AC, Pereira L, Rocha-Santos TAP, Vasconcelos MW, Roriz M, Rodríguez-Alcalá LM, Gomes AMP, Duarte AC. **2015**. Chemical composition of red, brown and green macroalgae from Buarcos bay in Central West Coast of Portugal. *Food Chemistry* 183, 197-207. *(FI (2014)=3.391; Rank=8/70 - Q1, Chemistry, Applied; Rank=8 /123 - Q1, Food Science & Technology; Rank=19/77 – Q1, Nutrition & Dietetics).*

- Chapter 4 describes the chemical composition and nutritive value of a selection of five edible and cultivated mushrooms. The selection of species, their elemental and proximate composition, fatty acids profile as well as FTIR-ATR characterization is deeply presented and discussed.

Chapter 4 is partially based on the following publication:

Rodrigues D, Freitas AC, Rocha-Santos TAP, Vasconcelos MW, Roriz M, Rodríguez-Alcalá LM, Gomes AMP, Duarte AC. ***2015***. Chemical composition and nutritive value of *Pleurotus citrinopileatus var cornucopiae; P. eryngii, P. salmoneo stramineus, Pholiota nameko* and *Hericium erinaceus. Journal of Food Science and Technology* 52(11), 6927-6939. *(FI (2014)=2.203; Rank=28/123; Q1, Food Science & Technology).*

- Chapters 5 and 6 report on the impact of enzyme, ultrasound and high hydrostatic pressure assisted extraction methods on biological properties of three selected seaweeds previously studied in chapter 3. The main effects of different aqueous-based extraction methods on proximate characterization and on several biological properties of the extracts are duly presented and discussed.

Chapters 5 and 6 are partially based on the following publications:

Rodrigues D, Sousa S, Silva A. G., Amorim M, Pereira L, Rocha-Santos TAP, Gomes AMP, Duarte AC, Freitas AC. **2015**. Impact of Enzyme- and Ultrasound-Assisted Extraction Methods on Biological Properties of Red, Brown, and Green Seaweeds from the Central West Coast of Portugal. *Journal of Agriculture and Food Chemistry 63* (12), 3177–3188. *(FI (2014)= 2.912; Rank= 2/56 - Q1, Agriculture, Multidisciplinary; Rank=11/70 - Q1, Chemistry Applied; Rank=13/123 - Q1, Food Science & Technology).*

Rodrigues D, Freitas AC, Queirós R, Rocha-Santos TAP, Saraiva JA, Gomes AMP, Duarte AC. 2016. Bioactive polysaccharides extracts from *Sargassum muticum* by high hydrostatic pressure. *Journal of Food Processing and Preservation*. Manuscript ID JFPP-10-15-1109 (*In press)*.

- Chapter 7 describes the impact of enzyme and ultrasound assisted extraction methods on biological properties of a cultivated mushroom previously studied in chapter 4. The main effects of different aqueous-based extraction methods on proximate characterization and on several biological properties of the extracts are duly presented and discussed.

- Chapter 8 presents a more thorough chemical and structural characterization of the four extracts with higher biological potential which were selected based on the results observed in chapter's 5 to 7. The chemical characterization of the extracts was based on analysis of elemental composition, amino acids and monosaccharides and the structural analysis was based on FTIR-ATR and ^{1}H NMR analysis.

Chapters 7 and 8 are partially based on the following publication:

Rodrigues D, Freitas AC, Sousa S, Amorim M, Vasconcelos MW, Costa J, Silva AMS, Rocha-Santos TAP, Duarte AC, Gomes AMP. **2016**. Chemical and estructural characterization of *Pholiota nameko* extracts with biological properties. *Food Chemistry*. Submitted, manuscript ID: FOODCHEM-D-16-01609.

- Chapter 9 compliments data presented in chapter 8, by presenting a deeper and broader biological characterization of the four selected extracts with higher functional potential. The biological characterization of the extracts was based on *in vitro* batch culture fermentation experiments conducted with faecal inoculum in order to observe changes in the main bacterial groups present in the microbiota and evaluation of anti-hypertensive activity.

Chapter 9 is partially based on the following publication:

Rodrigues D, Walton G, Sousa S, Rocha-Santos TAP, Duarte AC, Freitas AC, Gomes AMP. **2016**. *In vitro* fermentation and prebiotic potential of selected extracts from seaweeds and mushrooms. *LWT-Food Science and Technology*. Submitted, manuscript ID: LWT-D-16-00190.

- Chapter 10 reports the development of a new functional food based on a dairy spreadable cream with incorporated extracts based on the assessment of its biological and technological potential. Biochemical characterization, sensorial evaluation, characterization and assessment of *in vitro* stability of functional extracts as well as storage stability are presented and discussed.

A paper is being prepared with the main results of chapter 10 to be submitted soon in peer-reviewed international journals in the Science Citation Index (SCI®, Thomson ISI).

- Chapter 11 outlines the concluding remarks concerning the various experimental phases of this PhD work, highlighting the main achievements and discussing directions for future work.

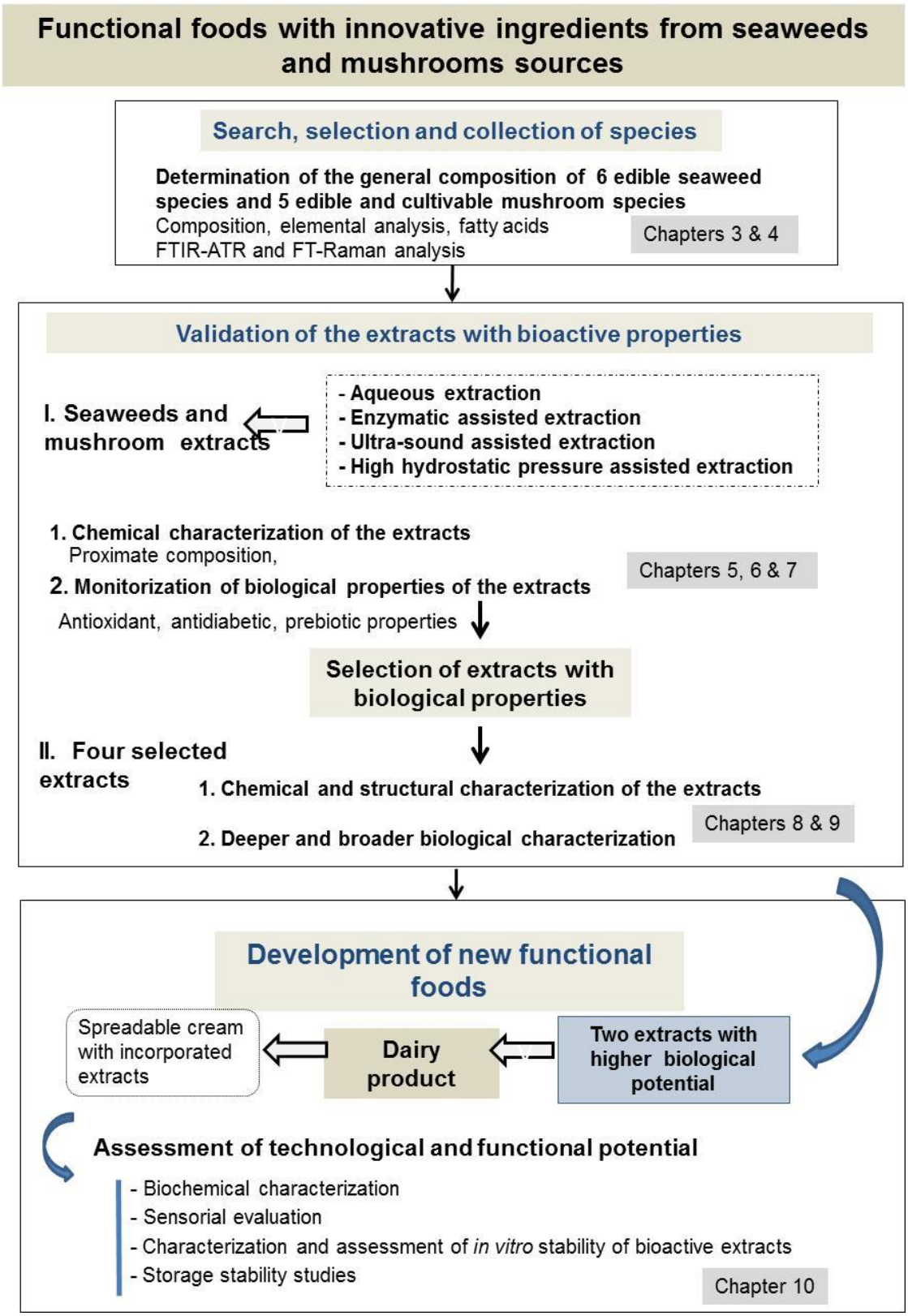

Figure 1.1. Outline of the developed experimental work.

Chapter 2

Introduction

2.1. General Overview

Consumers are becoming more health conscious and nowadays it is not possible to overlook the importance that diet can play in health/well-being. Consumption of healthy foods such as cereals, vegetables, fruits as well as marine foods rich in polyunsaturated fatty acids (PUFAs) beyond meeting basic nutritional needs, are also crucial for health promotion and/or maintenance as well as for disease risk reduction (Shahidi, 2009). The research and development of new functional foods (FF) has been the target of many studies over the last years. Nutritional science has been evolving from a focus on identifying nutrients and the respective amounts to prevent deficiency diseases to a focus on improving quality of life and health (Ramesh, 2012). According to several authors several foods can play an important role by preventing or hindering progression of chronic diseases such as atherosclerosis, obesity, diabetes, hypertension, osteoporosis, cancer and cardiovascular disease (Torres-Fuentes et al., 2015; Reedy et al., 2014; Saita et al., 2014; Streppel et al., 2014; Estruch et al., 2013) as well as relieving symptoms in osteoarthritic patients, for example (Messier et al., 2013). However, independent of a higher level of nutritional knowledge today's consumer and populations as a whole, still need to pinpoint strategies that may enable overcoming modern age diseases such as those previously mentioned. For example, the positive role of FF and nutraceuticals in the primary prevention of cardiovascular diseases was recently reviewed by Alissa & Ferns (2012). Based on various criteria, there has been a growing interest in research/development/commercialization of FF.

Functional foods belong to foods that besides their nutritional effects, have demonstrated benefit to one or more functions of the human organism, improving the state of health or well-being, and reducing the risk of disease (Plaza et al., 2008). In this context, it is important to take into account some important aspects that condition the effectiveness of the rationale upholding FF. Firstly, a functional effect of a food is distinct from the nutritional effect of the same food. Furthermore, its biological efficacy must be proven satisfactorily by demonstrating how effectively the functional food can promote an improvement of a physiological function or contribute to a reduction of risk of developing a pathological process. Functional foods also need to promote their beneficial action at the normal consumed doses (Plaza et al., 2008).

Functionality of a food may be inherent to a feature introduced by innovative extracting or processing technologies of the ingredient (Biström & Nordström, 2002), or may result from the addition of existing or new health-promoting food ingredients or probiotic microorganisms to the food matrix increasing their concentration, their bioavailability or their stability (Biström & Nordström, 2002).

The strong development of FF as a method to improve or maintain health is currently driving an intense field of research in the exploration of new compounds with real health effects. Sources of functional food ingredients (FFI) exist in many different reservoirs that may be found both in terrestrial and marine environments. Marine resources have numerous compounds with nutraceutical value to be used as FFI: omega-3 oils, chitin, chitosan, fish protein hydrolysates, algal

constituents, carotenoids, taurine and other bioactive compounds (Kadam & Prabhasankar, 2010) that can be added at different stages, from processing to storage, of the food production process. Although many marine functional ingredients are presently known, it is believed that many additional marine ingredients remain to be evaluated and new sources are yet to be discovered. On the other hand, terrestrial sources, although more heavily explored, as is the case with botanical bioactives given the historical interest from the pharmaceutical industry and more recently from the nutraceutical and food industries, still have less explored reservoirs of great interest in this dominion, such as mushrooms.

From a marine perspective, algae, in particular, edible seaweeds are a very interesting natural source of compounds with biological activity that could be used as functional ingredients. Considering their great taxonomic diversity and the research on identification of biologically active compounds they can be seen as a vast untapped resource. Seaweeds are well-known for their low calorie content, and for their richness in polysaccharides, minerals, vitamins, proteins, particularly the high contents found in red algae, steroids, lipids with long-chain PUFAs, particularly those of red and brown algae, dietary fibres, contributing positively to intestinal transit time and cholesterol metabolism (Cabral et al., 2011; Lordan et al., 2011), and nutritional properties which make them increasingly sought for commercial purposes.

In addition to their valuable nutrients, the presence of bioactive molecules such as polyphenols, conjugated fatty acids, sterols and pigments (for example carotenoids) target seaweeds to be incorporated in the diet as foods because they are claimed to contain low cholesterol content, fight obesity, reduce blood pressure, tackle free radicals and promote healthy digestion (Plaza et al., 2008); furthermore, some of them have demonstrated biological properties, such as antibacterial, antiviral, anticoagulant and antioxidant properties as well as playing important roles in the prevention of neurodegenerative diseases (Ibañez & Cifuentes, 2013).

The biological importance of seaweeds can be found in recent reviews (Kim et al., 2015; Lange et al., 2015; Pádua et al 2015; Domínguez, 2013a). An exhaustive description of bioactive compounds from seaweeds and their application in FF as well as emerging legislation issues is reported by Holdt & Kraan (2011) and updated by Domínguez (2013b) and more recently by Kim & Chojnacka (2015). The perspective of seaweeds as a sustainable functional food for complementary and alternative therapy has been reviewed by Mohamed et al. (2012).

As a terrestrial reservoir, mushrooms are also of tremendous potential interest to research FFI since they can provide a set of ingredients with nutritional and health benefits. Mushrooms are a source of protein, fibre, vitamins and minerals and, above all, of bioactive compounds such as phenolic compounds, polysaccharides, terpenes and steroids (Moon & Lo, 2013). Hence, besides being part of the human diet and duly appreciated for their sensory characteristics, mushrooms are also used as a medicinal source (Tsai et al., 2009; Wong & Chye, 2009). Different bioactive compounds from edible mushrooms were reported to possess antioxidant (Heleno et al., 2015a; Lin et al., 2015; Pattanayak et al., 2015), antitumor (Liu et al., 2015; Zheng et al., 2015), antimicrobial (Heleno et al., 2015b; Smolskaitė et al., 2015), immunomodulatory (Wu et al., 2014;

Lin et al., 2013), antiatherogenic (Mori et al., 2008) anti-inflammatory (Taofiq et al., 2015; Silveira et al., 2014) and cardiovascular diseases preventive roles (Guillamón et al., 2010). Lentinan and schizophyllan are examples of fungal polysaccharides that belong to the group of so-called biological-response-modifiers many of which have antitumor properties (Giavasis, 2014). Furthermore, fungal oligosaccharides are potential sources to demonstrate prebiotic properties. According to Aida et al. (2009) potential prebiotics from mushrooms should be researched upon since consumers could benefit simultaneously from the prebiotic effect of the mushroom extract itself coupled to other possible medicinal effects. The biological importance of mushrooms and their properties continue to be subject of interest, easily reflected in the several reviews that have emerged recently (Ruthes et al., 2016; Lau et al., 2015; Zhu et al., 2015; El Enshasy & Hatti-Kaul, 2013).

Based on the above considerations, seaweeds and mushrooms do represent a tremendous potential for many novel healthy FFI and biologically active compounds constituting a research area with much to explore for food purposes. However, despite the scientific interest for the use of marine and mushroom functional ingredients, there are various challenges that still need to be overcome before they may be used in the development of new FF or nutraceuticals; examples of such challenges include the application of efficient extraction methods as well as an effective characterization procedure, in order to obtain food grade validated extracts with biological properties.

Isolated FFI need to rely upon food compatible methods with economically viable yields and therefore different extraction methods must be applied in order to maximize the extraction efficiency of FFI with biological properties. The correct adjustment of parameters, such as temperature, pH, and extraction time, greatly influences the yield and prevents the possible structural alteration of the sulphated polysaccharides (Hahn et al., 2012).

2.2. Functional ingredients from seaweeds and their biological properties

As previously mentioned seaweeds in their great taxonomic diversity represent a natural treasure source of new compounds with bioactive properties. These can be used as FFI and nutraceuticals for potential beneficial health effects and prevention of various diet-related chronic diseases.

The rapid growth cycle associated with many of the species of marine macroalgae (seaweeds), their cultivation and the possibility of controlling the production of several bioactive compounds by manipulating the cultivation conditions are important aspects that enable these organisms to be considered as genuine natural reactors being, in some cases, a good alternative to the chemical synthesis of such compounds (Plaza et al., 2008). Production of valuable compounds or metabolites by seaweeds at a sustainable level can be optimised using different approaches. Their metabolism can be influenced by parameters such as water temperature, salinity, light and nutrients, so by manipulating these conditions their bioreactor properties will provide different types of compounds at different quantities. Some seaweeds live in complex

habitats and are subject to extreme conditions being forced to quickly adapt to such new environmental conditions and in order to survive they produce a wide variety of biologically active secondary metabolites (Plaza et al., 2008) like acetogenins, terpenes, derivatives of amino acids, simple phenols and polyphenols, substances that in general differ from plant products because they are often halogenated (de Carvalho & Roque, 2000) and for that, research on identification of biologically active compounds from seaweeds can be seen as an almost unlimited field. Gathering such information on metabolite production in natural habitats is crucial because it can then be used to optimise cultivation conditions in artificial production systems. These human-controlled cultivation systems add value in that cultivation conditions or stress application conditions are better controlled (light, temperature, salinity, nutrients availability) and sustainable high levels of productivity all year-round are assured (Ibañez & Cifuentes, 2013). According to Chojnack et al. (2012), there are about ten thousand identified species of seaweeds and only about 5% of them are used as food for either humans or animals. The multitude of different morphological types is accounted for by their complexity, structure and environmental adaptation (Pereira, 2015a). More than one hundred seaweed species are used worldwide, especially in Asian countries, including China and Japan as major producers, as sea vegetables (Fleurence et al., 2012). In the South of Europe the use of edible seaweeds for food purposes is still residual, not fitting in with the regular consumption habits. Therefore, the extraction of compounds with biological interest from seaweeds and their incorporation in foods matrices may be an alternative way to introduce these beneficial candidates in the healthy eating habits of the western world. According to Lordan et al. (2011) the ingestion of such compounds has the advantages of presenting a great bioavailability, being of natural origin and furthermore, isolation does not require a high cost and more importantly they affect positively some pathologies. All algae do not need the same light intensity to perform photosynthesis so the phyla and classes of macroalgae are generally defined by the presence of specific pigments being classified as brown algae (phylum Heterokontophyta or Ochrophyta), red algae (phylum Rhodophyta), or green algae (phylum Chlorophyta) (Lordan et al., 2011; Kilinç et al., 2013).

Green seaweeds possess chlorophyll a, b and carotenoids. Chlorophyll a is the pigment responsible for their green colour and appearance similar to land plants. This pigment is essential for photosynthesis, which requires great amounts of light and therefore, green seaweeds cannot be found at deep and shadowed places. It gives them an advantage, the ability to live higher up shore without competition from the red or brown seaweeds. Green seaweeds are found on both sandy and rocky beaches. Many can tolerate low salinity and will colonize areas where rivers meet the sea. Some green seaweeds such as *Codium* and *Ulva* (formerly *Enteromorpha*) are commonly used as food sources.

Brown seaweeds are often found on rocky intertidal shores. Their pigments are the chlorophylls a, c, and carotenoids, particularly fucoxanthin, responsible for the brown colour (Pereira, 2009). This group of seaweeds includes species such as the giant kelp *Macrocystis* or the invasive seaweed *Sargassum.* Similarly to the green and the red seaweeds, the brown seaweeds

may be used as a food source and can be consumed by humans in the edible raw, dried or cooked forms. Brown seaweeds are also harvested for industrial and pharmaceutical uses (Chapman, 2013, Kilinç et al., 2013).

Red seaweeds have had a more diverse evolution than the green and the brown counterparts. Many species cannot stand desiccation and dominate the intertidal rock pools. Others tolerate desiccation, such as the red alga *Porphyra* spp. which can often be seen stretched out like a dry black film over mussel beds on rocky beaches. Red seaweeds exhibit a broad range of morphologies, simple anatomy and display a wide array of life cycles. Their pigments are chlorophyll a, phycobilins and carotenoids including β-carotene, lutein and zeaxanthin (Pereira, 2015b). About 98% of the species are marine, 2% freshwater and a few rare terrestrial/sub-aerial representatives (Pereira, 2012). Some species such as *Gelidium* and *Gracilaria* are used in the manufacture of agar and others, like *Eucheuma* and *Kapaphycus alvarezzi,* are used in the production of carrageenan.

2.2.1. Polysaccharides

Phycocolloids are structural polysaccharides with high molecular weight found in the cell wall and intercellular spaces of freshwater and marine macroalgae (Figure 2.1). These compounds usually form colloidal solutions which give these polysaccharides the ability to be used as thickeners, gelling agents and stabilizers for suspensions and emulsions in diverse industries (Pereira et al., 2015b). The total concentration of polysaccharide in the seaweeds can range from 4% to 76% of dry weight (Holdt & Kraan, 2011) depending on the seaweeds species, geographical origin, season, water temperature, place and method of cultivation, harvesting time among other factors (Lordan et al., 2011; Synytsya et al., 2010). Agar, carragenaans and alginates are examples of polysaccharides derived from red and brown seaweeds, respectively (Figure 2.1) that are widely used as stabilizers and thickeners in gels as well as in foods (Rasmussen & Morrissey, 2007). Other less predominant polysaccharides of interest are fucoidans (brown seaweeds), xylans (certain red and green seaweeds) and cellulose (in all genera). Seaweeds also contain storage polysaccharides such as laminarin (brown seaweeds) and floridean starch (red seaweeds) (Figure 2.1) (Herrero et al., 2013). Furthermore, sulphated fucans, such as ulvans from green seaweeds, carrageenans from red seaweeds and fucoidans from brown seaweeds are also present and of particular biological importance.

Most of these polysaccharides are not digested by humans and can be regarded as dietary fibres, both soluble and insoluble type, for human nutrition associated with reductions in plasma cholesterol and glucose levels (viscosity-mediated effects) or reduced intestinal transit time (fecal bulking effect). Notwithstanding, they are being further evaluated as new possible prebiotic compounds given the susceptibility of some of these soluble polysaccharides to fermentation by the colonic microbiota; the use of algal fibre promotes the control and maintenance of intestinal microbiota and the production of short chain fatty acids thereby (Gupta & Abu-Ghannam, 2011).

For example, sulphated polysaccharides can act also as prebiotics exerting growth-promotion of beneficial bacteria in the gastro-intestinal tract imparting health-improving benefits to the host (Holdt & Kraan, 2011).

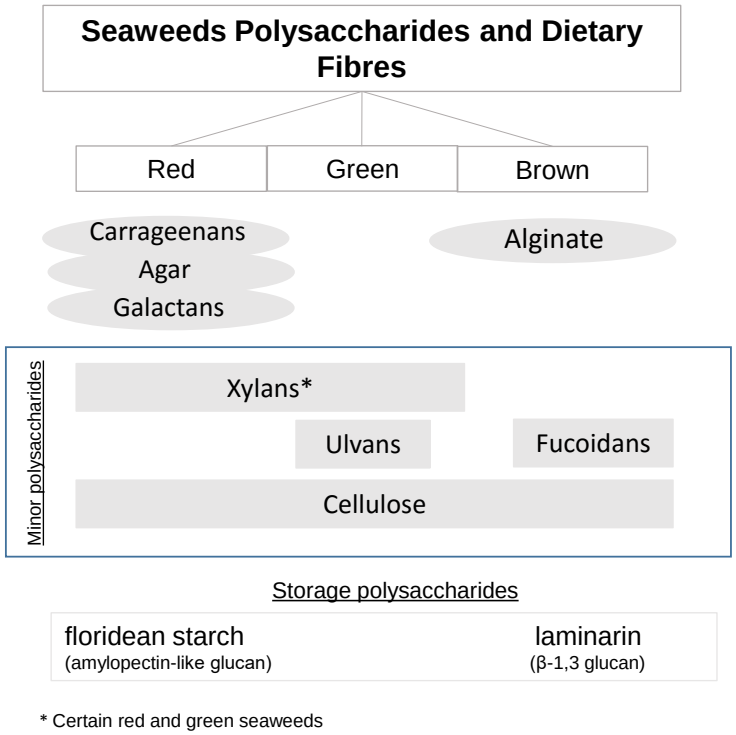

Figure 2.1. Schematic representation of the main polysaccharides and dietary fibres in seaweeds, based on Herrero et al. (2013).

Moving on to a more specific characterization of the different seaweed polysaccharides, and of particular biological importance, are the sulphated polysaccharides, of which seaweeds are a major source; their variable structure is correlated with the seaweed species. Sulphated polysaccharides found in red seaweeds are mainly galactans consisting entirely of galactose or modified galactose units, whereas in brown seaweeds they are fucans, which comprise families of polydisperse molecules based on sulphated L-fucose; heterofucans are also called fucoidans. In turn, the major water-soluble polysaccharides found in green seaweed are ulvans (Jiao et al., 2011); some of them are a significant source of sulphated galactans particularly in *Codium* species (Chlorophyta, green seaweeds) (Farias et al., 2008; Matsubara et al., 2001). Sulphated galactans are widely used in food industry due to their jellifying and thickening properties (McHugh, 2003).

Sulphated polysaccharides from seaweeds have been a target for a lot of research over the last years because of their potential in terms of biological properties; anticoagulant, antithrombotic, antioxidant, antitumor, anti-inflammatory, antiviral, anti-proliferative, anti-peptic and anti-adhesive activities have been associated to these compounds (Wijesekara et al., 2011; Pomin & Mourão, 2012) and have recently been reviewed by Ngo & Kim (2013) for all seaweeds, in general, and by Wang et al. (2014) for green seaweeds, in particular.

Carrageenan and xylomannan sulphate are other sulphated polysaccharides that are found in some red seaweeds and which possess antiviral properties (Ray et al., 2015; Leibbrandt et al., 2010). The carrageenan hydrocolloid is a linear sulphated polysaccharide of D-galactose and 3,6-anhydro-D-galactose extracted from some red seaweeds species. There are three general forms (kappa, lambda and iota) but, in general, seaweeds do not produce pure carrageenans, but more likely a range of hybrid structures (Pereira et al., 2009; Prado-Fernández et al., 2003). Each of the copolymers kappa and iota carrageenan, jellify differently and are used in food production due to their excellent physical and functional properties (thickener, jelling and stabilizer) in products such as yogurt, puddings, ice cream, sausages, hams, jams among others. They also possess several applications in non-food industries as well as in the production of pharmaceutical products with anti-coagulant and anti-inflammatory properties (Cardozo et al., 2007).

Fucoidan is a type of complex sulphated polysaccharide that contains α-1,3-linked sulphated L-fucose as its main sugar unit and sulphate ester groups (Synytsya et al., 2010). Antitumor and anti-angiogenic activities are affected increasingly by the number of sulphate groups in the fucoidan molecule (Koyanagi et al., 2003) but fucoidan has also been reported to possess diverse biological activities such as anticoagulant, antioxidant (Wang et al., 2010), antitumor, anti-inflammatory, and antiviral activities (Cumashi et al., 2007). A review focusing fucoidan as a natural bioactive ingredient for FF was recently published by Thanh-Sang & Kim (2013).

Agar is a water soluble long chain polysaccharide which dissolves or disperses in water. Agar may be extracted from species such as *Gracilaria* and *Gelidium* and is widely used in the manufacture of gelatin. Agar consists of two fractions: the agarose and agaropectin, considered a complex mixture of polysaccharides; the agaropectin has low gelling power (Holdt & Kraan, 2011). Agar-agar has been reported to decrease concentration of blood glucose, to exert an anti-aggregation effect on red blood cells, and to have antioxidant, antitumor as well as antiviral activities (Holdt & Kraan, 2011).

Laminarin is one of the major polysaccharides found in brown seaweeds which have been identified as a modulator of intestinal metabolism through its effects on intestinal pH, mucus composition and short chain fatty acid production (O'Sullivan et al., 2010; Devillé et al., 2007) with antiviral and antibacterial properties. Antioxidant activity of laminarin depends on its molecular weight and chemical structure. Its chemical structure consists of β-(1→3)-linked glucose in the main chain and random β-(1→6)-linked side-chains (Brown & Gordon, 2005) and their content in seaweeds is about 10% of dry weight, but can seasonally reach up to 32% (Holdt & Kraan, 2011).

Alginates can be extracted from brown seaweeds; they are important soluble fibres due to their biodegradability, immunogenicity and ability to form gel with a variety of cross-linking agents being used in several fields like food industry, medicine, and biotechnology (Lee & Mooney, 2012)). The acid form (alginic acid) is a polymer consisting of two types of hexuronic acid monomers linked by 1→4 bonds: β-D-mannuronic acid and α-L-guluronic acid, respectively (Andriamanantoanina & Rinaudo, 2010). This polysaccharide has shown antitumor, anticoagulant and antiviral activities; furthermore evidence shows its capacity to prevent obesity as well as large intestine cancer and

diabetes and to reduce low-density lipoprotein (LDL)-cholesterols in rats (Ghosh et al., 2009; Wang et al., 2008; Athukorala et al., 2007; Amano et al., 2005). Alginic acid can also apparently reduce hypertension and porphyran from red seaweeds has been related with immune-regulatory, antioxidant and antitumor activities (Mohamed et al., 2012).

Besides the previously mentioned anticoagulant (de Zoysa et al., 2008; Mao et al., 2006), anti-inflammatory (Lee et al., 2013), antiviral (Hayashi et al., 2008; Mandal et al., 2007) and antitumor activities (Synytsya et al., 2010) of fucoidans and alginic acid derivatives produced by brown seaweeds (*Ecklonia cava*, *Ascophylum nodusum* and *Undaria pinnatifida*, besides other species) these have also been demonstrated to play an important role as free-radical scavengers and antioxidants preventing oxidative damage (Vijayabaskar et al., 2012). Evidence from different studies suggested that the antioxidant and anticoagulant activities of polysaccharides were strongly dependent on the content of sulphate groups, the molar ratio of sulphate/fucose and sulphate/total sugar, and the molecular weight (Wang et al., 2010).

A summary of potential biological properties and food applications for seaweeds' polysaccharides is displayed in Figure 2.2.

2.2.2. Proteins, peptides and amino acids

The role of protein in the diet has been acknowledged worldwide and currently dietary proteins are one of the major sources of bioactive compounds with beneficial physiological impact on human and animal health (Freitas et al., 2013). In general, protein content in seaweeds will vary according to the geographical location, harvest season and the species; higher contents are found in green and red seaweeds (10-47% of the dry weight) in comparison to the brown seaweeds where contents are two-fold less (5-15% of the dry weight). As far as the season is concerned, higher protein contents have been reported during the winter and spring months whereas in the summer the lowest protein contents have been reported (Ibañez & Cifuentes, 2013).

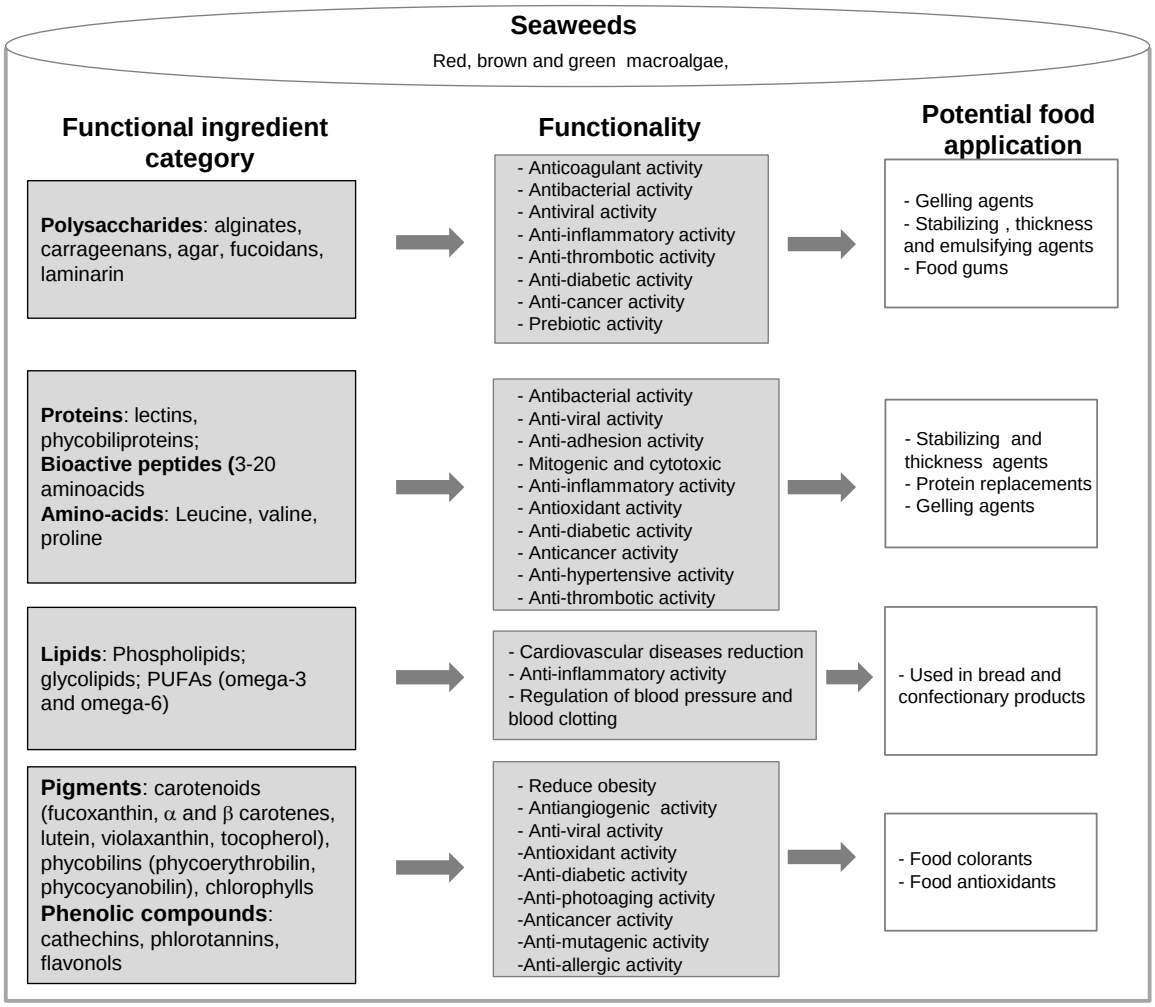

Figure 2.2. A general overview of potential biological properties and food applications of major functional ingredients from seaweeds.

Extraction of protein from most seaweeds is difficult due to the presence of large amounts of polysaccharides in the cell wall such as alginates in brown seaweeds or carrageenans in red seaweeds. Enzymatic hydrolysis by digestive enzymes or by proteolytic microorganisms has been a successful strategy to enhance extraction of peptides from seaweeds (Wijesinghe & Jeon, 2012). Some of the important bioactive proteins that can be extracted from seaweeds are lectins and phycobiliprotein (Aneiros & Garateix, 2004). A summary of potential biological properties and food applications for seaweeds' proteins and derived products (products and amino-acids) is displayed in Figure 2.2.

Seaweeds lectins have been detected and isolated from the red seaweeds; the lectins are generally bound to polysaccharides and participate in many biological processes such as intercellular communication and have antibacterial, antiviral or anti-inflammatory activities (Chojnacka et al., 2012). Bioactive lectins are found in species such as *Ulva* sp. (Chlorophyta), *Eucheum*a sp. and *Gracilaria* sp. (Rhodophyta) and have biotechnological applications in several scientific and medicinal fields of research (Holdt & Kraan, 2011). Bioactive properties like mitogenic, cytotoxic, antibiotic, anti-nociceptive, anti-HIV, anti-inflammatory and anti-adhesion

activities has been reported for lectins (Mori et al., 2008; Smith, 2004). In turn, phycobiliproteins are oligomeric proteins, built up from two chromophore-bearing polypeptides found in seaweeds which have antioxidant, antidiabetic and anticancer activities (Fitzgerald et al., 2011; Aneiros & Garateix, 2004).

Bioactive peptides usually contain 3-20 amino acid residues and their activities are based on their amino acid composition and sequence and they are related with some biological functions such as antihypertensive, immune modulator, antioxidant, anticancer, antithrombotic and antimicrobial activities (Lordan et al., 2011; Kim & Wijesekara, 2010). Suetsuna et al. (2004) isolated 10 dipeptides from seaweed *Undaria pinnatifida* (Phaeophyceae) with four of these dipeptides containing the greatest ACE-inhibitory potential; their hypotensive effects were investigated *in vivo* and their results demonstrated a decrease of blood pressure in hypertensive rats.

From a nutritional quality standpoint most edible seaweeds contain amino acid profiles with a representative fraction of all the essential amino acids ranging between 36.3 and 57.7% of total amino acids (Yuan, 2008). Most seaweed species are a rich source of the acidic amino acids. Brown seaweeds are a rich source of threonine, valine, leucine, lysine, glycine, cysteine, methionine, histidine among others (Holdt & Kraan, 2011). In the majority of the peptide sequences in antioxidant marine-derived peptides, the presence of hydrophobic amino acids such as leucine, valine, and proline is common which contributes greatly to their potential antioxidant activity (Mendis et al., 2005) and have a major role in the inhibition of lipid peroxidation (Kumar et al., 2011).

2.2.3. Lipids and fatty acids

Phospholipids and glycolipids are the major classes of lipids found in seaweeds accounting for only 1-5% of cell composition (Chojnacka et al., 2012). Since at lower temperatures seaweeds are able to accumulate PUFAs, species found in cold regions contain more PUFAs than those in moderate climates. PUFAs are known to regulate a variety of body functions such as blood pressure, blood clotting, proper development and function of brain and nervous system (Wall et al., 2010), and also play an important role in regulating the inflammatory response through the production of inflammatory mediators termed eicosanoids (Calder, 2006). PUFAs account for almost half of the lipid fraction, with much of it occurring in the form of n-3 (omega-3) and n-6 (omega-6) fatty acids such as eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic acids (ARA) (MacArtain et al., 2007). The predominant fatty acid in various seaweeds is EPA (C20:5, n-3), which may be found at concentrations as high as 50% of the total fatty acid content (Holdt & Kraan, 2011). Red and brown seaweeds are rich in the omega-3 fatty acids, (EPA and α-linolenic acid), omega-6 fatty acids (ARA and α-linoleic acid), along with relatively high levels of oleic and palmitic acids

(Dawczynski et al., 2007) whereas green seaweeds are more characterized by the presence of hexadecatetraenoic, oleic and palmitic acids (Figure 2.2).

From a nutritional standpoint it is most important that an appropriate balance between omega-3 and omega-6 is maintained since these fatty acids (FAs) work together to promote health; most omega-6 FAs (precursors of arachidonic acid and prostaglandin E2) tend to promote inflammation and tumour growth (Simopoulos, 2001), whereas omega-3 FAs antagonize such physiological phenomena; hence the intake of seaweeds rich in omega-3 FAs would help to achieve this balance. Van Ginneken et al. (2011) studied the efficiency of using seaweeds as a source of PUFAs and have found a very low content of omega-6 FAs (only 3%) and therefore a very low omega-6:omega-3 ratio even below that indicated by experimental evidence as being optimum from a body homeostasis point of view (ratios of 4:1-5:1 and no higher than 10:1 are suggested); this achievement is important and supports evidence that seaweeds lipids contents could help in the prevention of inflammatory, cardiovascular and neural disorders.

2.2.4. Pigments

The biological importance of seaweeds and their pigments can be found in Gamal (2010) and Pangestuti & Kim's (2011) reviews. Carotenoids are a group of pigmented compounds which act as a light energy harvester or as a stabiliser of protein folding in the photosynthetic apparatus of photosynthetic marine organisms and are potent antioxidants that inactivate reactive oxygen species (Figure 2.2). Carotenoids may also be used as food colourings, feed supplements, nutraceuticals and in pharmaceutical and cosmetic products. Carotenoids are in the basis of the seaweed classification system and include for example fucoxanthin and β-carotene in brown seaweeds; β-carotene, lutein, neoxanthin and violaxanthin green seaweeds, and β and α-carotene, lutein and zeaxanthin in red seaweeds (D'Orazio et al., 2012; Larsen et al., 2011; Cornish & Garbary, 2010). Some of the most important carotenoids are β-carotene and fucoxanthin (Chojnacka et al., 2012). Fucoxanthin, one of the most abundant carotenoids with a unique structure including an allenic bond and a 5,6-monoepoxide in its molecule, is present in species of brown seaweeds such as *Undaria pinnatifida* (Wakame), *Hijikia fusiformis* (Hijiki), *Laminaria japonica* (Ma-Kombu) and *Sargassum fulvellum* (Phaeophyceae). It has been indicated to reduce obesity by inhibiting intestinal lipase activity (Matsumoto et al., 2010) as well as to possess antiangiogenic activity by suppressing the differentiation of endothelial progenitor cells into endothelial cells involved in new blood vessel formation and anti-viral properties (Cornish & Garbary, 2010). Fucoxanthin has also been shown to have an important antioxidant activity, as well as antidiabetic, anti-photoaging and anticancer properties; these and other health properties of fucoxanthin have been very recently reviewed by Gammone & D'Orazio (2015) with a special focus on the anti-obesity activity and by Zhang et al. (2015).

Xantophylls, another type of carotenoids presenting one or more functional groups containing oxygen, are the main pigment found in brown seaweeds, namely in *Undaria pinnatifida* and have

been related with some activity against cerebrovascular diseases (Ikeda et al., 2003). Overall, the biological functions and commercial applications of marine carotenoids can be found in Vílchez et al. (2011) review; more recently Gammone et al. (2015) reviewed the role of marine carotenoids against oxidative stress and their potential applications in preventing and treating inflammatory diseases.

Phycobiliproteins are classified as protein-pigment complexes (red, phycoerythrobilin, purple-deep blue, phycocyanobilin) found in marine macroalgae which are used as natural colour pigments in food products (Rasmussen & Morrissey, 2007).

Chlorophylls are green pigments that are used as natural colorants for foods and beverages in the food industry (Rasmussen & Morrissey, 2007) and have been shown to possess some biological activity concerning anticancer properties. In an era where the trend for natural is prevailing in food product development the use of these compounds as natural colorants instead of synthetic counterparts is an effective alternative, contributing simultaneously to consumer's well-being and health promotion.

2.2.5. Phenolic compounds

Polyphenols or phenolic compounds are found at high levels in seaweeds and are associated with very strong antioxidant properties as well as other biological activities such as anti-inflammatory or anti-mutagenic properties (Figure 2.2). In seaweeds the content and profile of these compounds vary with the species and are associated to chemical defences against external conditions such as stress and herbivores (Li et al., 2011). Seaweeds species such as *Undaria* sp., *Laminaria* sp. and *Fucus* sp. (Phaeophyceae, brown seaweeds), *Porphyra* sp. (Rhodophyta, red seaweeds) are rich sources of polyphenolic compounds (Lordan et al., 2011). For example, in brown seaweeds the major phenolic compounds are the phlorotannins such as fucols, phlorethols, fucophlorethols, fuhalols and halogenated and sulphited phlorotannins (Ibañez & Cifuentes, 2013). These phlorotannins are highly hydrophilic compounds with antioxidant, anti-inflammatory, anti-diabetic, antitumor, antihypertensive and anti-allergic activities; furthermore, they have been demonstrated as inhibitors of hyaluronidase and of metalloproteinases enzymes, two virulence factors that may be expressed by some microorganisms (Ngo et al., 2011; Thomas & Kim, 2011).

In red seaweeds the phenolic compounds rutin, hesperidin, morin, catechol, catechin and epigallocatechin gallate have been linked with anti-inflammatory activity, most probably due to the attenuation of the production of nitric oxide.

Phlorotannins, cathechins and flavonols have all been explored as FFI (Larsen et al., 2011; Cofrades et al., 2010).

2.2.6. Minerals

It is well-known that seaweeds are recognized to be rich in minerals (Ca, Mg, Na, P and K) and trace elements (Zn, I and Mn) which is related to their capacity to retain inorganic marine substances due to polysaccharides in the cell surface, accounting for up to 36% of dry matter in some species (Lordan et al., 2011). Similar to the protein content previously discussed, seaweed mineral content also varies with the seasons; higher in winter months and lower in summer months. They are one of the most important sources of Ca and K (MacArtain et al., 2007) and some seaweeds are indicated to be used as food supplements to meet the recommended daily intake of some minerals and trace elements.

Selenium, I, as well as Zn and Mg are other minerals that are more abundant in some seaweeds than in other foods, for example meats (Larsen et al., 2011). Increased dietary intake of selenium has been related to protection against several cancers. In some countries or populations with low iodine intake, seaweeds, which are one of the best natural sources of iodine (Gupta & Abu-Ghannam, 2011) are an alternative way to deliver iodine through diet to maintain thyroid function instead of the iodine fortified salt.

2.3. Functional ingredients from mushrooms sources and their biological properties

According to Sabaratnam et al. (2011), mushrooms have been used by humans since thousands of years for food and/or medicine applications. They are receiving an increasing attention in the last years from the researchers who began to realize the potential beneficial effect of proteins, carbohydrates, fats, and others compounds on the health of individuals (Wani et al., 2010). As previously discussed, mushrooms are of important nutritional value constituting a great source of protein (20-25%), dietary fibre (13-24%), vitamins (B1, B2, B12, C, D, and E), minerals (selenium and potassium), carbohydrates (37-48%) (Sabaratnam et al., 2011; Alam et al., 2008) and of some secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids (Cheung et al., 2003). They are also low in fats (4-5%) (Sabaratnam et al., 2011; Alam et al., 2008) and provide low energy content.

For many years mushrooms have been recognized for their medicinal effects due to their antifungal, antibacterial, antioxidant, antimicrobial and antiviral properties, properties which have also enabled their use as FF (Wani et al., 2010). Due to the presence of different bioactive substances mushrooms have been used in several medical therapies, including antitumor, immune-modulation, anti-diabetic treatments (Cheng et al., 2012) and are reported to play a preventive role in some cardiovascular diseases (Guillamón et al., 2010) and anti-inflammatory and analgesic properties (Smiderle et al., 2008).

Edible mushrooms contain many antioxidant compounds such as carotenoids, flavonoids, phenolic compounds, polysaccharides and antioxidant enzymes (Vaz et al., 2010). Among all the

bioactive compounds of mushroom origin, polysaccharides are the most extensively researched. Polysaccharides isolated from different mushrooms have been shown to have antitumor, cholesterol-lowering and immunity, antioxidant, antimicrobial, anticoagulant and anti-thrombotic activities (Sharma et al., 2015; Souza et al., 2015; Giavasis, 2014; Giri et al., 2012; Reis et al., 2012; Yildirim et al., 2012; Synytsya et al., 2010).

Bioactive proteins constitute another important fraction of functional compounds in mushrooms. They produce a large number of proteins and peptides with interesting biological activities, such as lectins, fungal immune-modulator proteins (FIP), ribosome inactivating proteins (RIP), antimicrobial proteins, ribonucleases, and laccases (Roupas et al., 2012; Xu et al., 2011).

Although several hundreds of species of edible mushrooms exist in the wild, less than 20 species are used extensively as food and only 8 to 10 species are regularly cultivated to a significant extent. The most commonly eaten species are *Agaricus bisporus* 'Paris mushroom' and *Lentinula edodes* (Shiitake Mushroom) which is the second most cultivated mushroom in the world (Ghorai et al., 2009). *Agaricus bisporus* has been identified as a natural antimicrobial source against tested organisms and has also been reported to have antioxidant activity (Abah & Abah, 2010). Shiitake's protein has a full battery of essential amino acids so it can be used extensively in a vegetarian diet. Its active ingredient, lentinan (a polysaccharide), has been shown to reduce cancer and cholesterol. The 'Shiitake mushroom' is as common in Asian countries as *Agaricus bisporus* is in the western world (Ghorai et al., 2009).

Mushrooms offer tremendous applications as they can be used as food and medicines representing one of the world's greatest untapped resources of nutrition and palatable food for the future (Wani et al., 2010).

A summary of potential biological properties and food applications for mushrooms' ingredients is displayed in Figure 2.3.

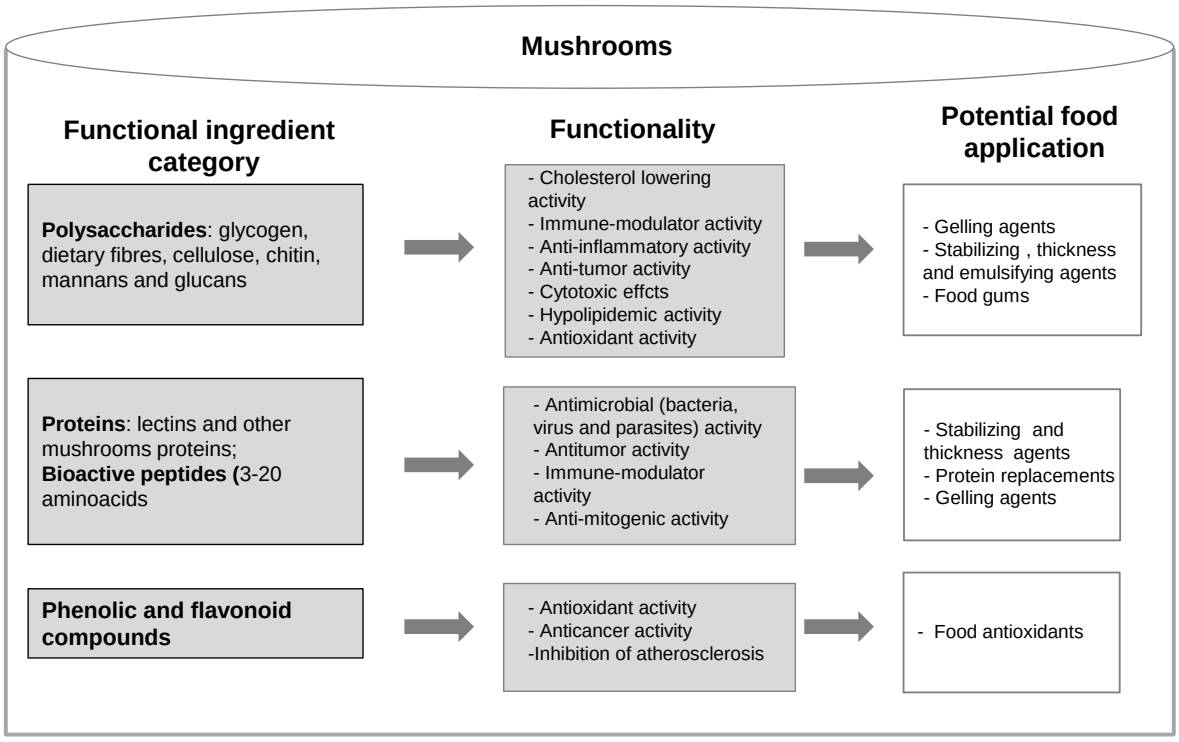

Figure 2.3. A summary of potential biological properties and food applications of major functional ingredients from mushrooms.

2.3.1 Proteins and polysaccharides

Mushrooms provide a set of ingredients with nutritional and health benefits. Protein is an important constituent of dry matter of mushrooms but not many of these proteins have been identified and even fewer characterized (Erjavec et al., 2012). It is known that mushrooms produce many bioactive proteins and peptides which are protective in function to the fungi themselves, primarily including lectins, fungal immunomodulatory proteins, ribosome inactivating proteins, antimicrobial/ antifungal proteins, ribonucleases and laccases (Xu et al., 2011). Lectins, for example, are nonimmune proteins that have exhibited highly potent antiproliferative activity toward some tumour cell lines (Xu et al., 2011) and have also revealed a potent anti-mitogenic activity on mouse splenocytes (Pohleven et al., 2009) (Figure 2.3). Mushroom aqueous extracts and proteins isolated from mushrooms have been shown to possess antibacterial (Zheng et al., 2010), antiviral (Wong et al., 2010), antiparasitic (Bleuler-Martinez et al., 2011), antitumor and immunomodulatory (Lindequist et al., 2005), as well as insecticidal (Pohleven et al., 2011) and nematotoxic activities (Zhao et al., 2009). The protein variability and limited characterization level reveals the great potential of higher fungi as a source of novel proteins (Erjavec et al., 2012).

Polysaccharides isolated from different mushrooms have shown a wide range of biological properties, including good cholesterol-lowering activity (Li et al., 2010) immunomodulatory, antitumor and anti-inflammatory activities (Li et al., 2008). As a result of the activation of the host's

immune system, these polysaccharides show significant antitumor, antiviral and antimicrobial activity, among other effects. Generally, bioactive polysaccharides from mushrooms consist of a β-linked glucose backbone displaying different patterns and degrees of branching. Fungal polysaccharides are represented by glycogen and such indigestible forms as dietary fibre, cellulose, chitin, mannans and glucans (Kim et al., 2003). Mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1→3), (1→6)-β-glucans and (1→3)-α-glucans but also as heteroglucans, here, side chains contain glucuronic acid, xylose, galactose, mannose, arabinose, or ribose as a main component or in different combinations (Wasser, 2002). Recently, several polysaccharides from mushrooms have been isolated and characterized, such as lentinan from *Lentinus edodes*, pleuran from *Pleurotus* mushrooms, schizophyllan from *Schizophyllum commune*, or ganoderan and ganopoly from *Ganoderma lucidum*. Molecular weight, primary structure, solution conformation and polymer charge are among the physico-chemical properties of polysaccharides that may determine their binding affinity to receptors as well as their effective biological properties.

From all of the polysaccharides isolated from mushroom origin, glucans are the most important and the most versatile due to their potent antitumor properties (Chakraborty et al., 2006). Evidences have shown that mushroom polysaccharides have direct and indirect antitumor activities, mostly by way of immunomodulation. Overall, β-glucans are responsible for anticancer, immunomodulating, anticholesterolemic, antioxidant, and neuroprotective activities of many edible mushrooms. They are also recognized as potent immunological stimulators, and their capacity for treating several diseases has been demonstrated. β-glucans bind to a membrane receptor and induce these biological responses (Valverde et al., 2015). Recent studies showed that polysaccharide isolated from the mycelium of the edible *Hericium erinaceus*, possessed various biological activities, exhibiting anticancer properties (Lee & Hong, 2010), immune stimulating, cholesterol-lowering (Luo & Chen, 2010; Lee et al., 2009) as well as cytotoxic, and hypolipidemic effects (Lee et al., 2009). These properties may contribute toward cardiovascular protection. The antioxidant properties of compounds found in *Pleurotus* sp. mushrooms continue subject of interest and target for studies (Mori et al., 2008). *Pholiota nameko* is also a widely studied mushroom; it has the potential to serve as an effective therapeutic agent for hyperlipidemic diseases, especially cardiovascular disease (Zheng et al., 2014; Li et al., 2010). However, much research is still needed to explore edible and cultivated mushrooms in terms of functional ingredients.

2.3.2 Other compounds

Edible mushrooms generally have a low lipid level; their overall content is hardly higher than 4-8% of the dry weight independently of the species. Low lipid content, among which linoleic (C18:2), oleic (C18:1) and palmitic (C16:0) are the major fatty acids, is one of the advantages of mushrooms as food suppliers. The presence of the PUFAs accounts for their contribution to the reduction of

serum cholesterol. The major sterol produced by edible mushrooms is ergosterol, which shows antioxidant properties (Vilaverde et al., 2015).

Mushrooms also contain various polyphenolic and flavonoid compounds, the bioactivity of phenolic compounds may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Yoon et al., 2011). Phenolic compounds are one of the groups of non-essential dietary components that have been associated with the inhibition of atherosclerosis and cancer and also have the ability to act as antioxidants acting as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers (Wong et al., 2009). The flavonoid compounds myricetin and catechin have been identified as the main flavonoids in mushrooms (Palacios et al., 2011); within the phenolic fraction protocatechuic, p-hydroxybenzoic, p-coumaric and cinnamic acids are among the phenolic compounds that have been identified (Heleno et al., 2012). Mushrooms are one of the best sources of vitamins especially providing a nutritionally significant content of B1, B2, B12, C, D, and E (Valverde et al., 2015). Higher dietary intake of mushrooms is associated with decreased breast cancer risk in both pre- and post-menopausal women and an additional decreased risk of breast cancer. Vitamin D2 could be one of the protective phytonutrients against breast cancer as mushrooms are rich in ergosterol as previously mentioned, generating vitamin D2 when exposed to ultraviolet B (UVB) light (Roupas et al., 2012).

The fruiting bodies of mushrooms are also characterized by a high level of well assimilated mineral elements. Major mineral constituents in mushrooms are K, P, Na, Ca, Mg and elements like Cu, Zn, Fe, Mo, Cd form minor constituents. K, P, Na and Mg constitute about 56 to 70% of the total ash content of the mushrooms with K being the most abundant (Wani et al., 2010). The mineral proportions vary according to the species, age, the diameter of the fruiting body and the type of the substrate.

2.4. Food compatible extraction methods

Extraction and isolation of compounds of interest from natural-derived products, able to be ingested or used for food purposes, need to rely upon compatible methods that are fast, environmentally friendly and with economically viable yields, yet such objectives do include several challenges. Seaweeds for example, are known to have a chemical and structural heterogeneous rigid wall which poses limitations to efficient extraction of the intracellular and wall compounds (Jeon et al., 2012). Several extraction approaches with seaweeds or mushrooms have been tested in the last years including pressurized water extraction (Palanisamy et al., 2014), supercritical fluid extraction (Mazzutti et al., 2012), ultrasound-assisted extraction (Kadam et al., 2013), micro-wave assisted extraction or extraction with different solvents such as methanol (Moro et al., 2012), ethanol and ethyl acetate (Seephonkai et al., 2012). Water-based extraction is food compatible, non-expensive and environment friendly but has low selectivity with low extraction efficiency (Herrero et al., 2006; Heo et al., 2003). Extraction based on several organic solvents has been attempted with higher selectivity and efficiency but some are non-food compatible such as hexane, diethyl ether, benzene

and acetonitrile for which solvent residues and environmental pollution are further drawbacks (Gil-Chávez et al., 2013; Domozych, 2011).

Enzyme-assisted extraction (EAE) has emerged with considerable interest because their hydrolytic action on natural-derived products, such as seaweeds or mushrooms, could weaken or disrupt cell wall structure and also breakdown complex interior storage compounds releasing intracellular compounds such as polysaccharides, peptides or amino acids (Wang et al., 2010). Algal cell walls are typically fibrous composites of microfibrillar polysaccharides embedded in a matrix of polysaccharides and proteoglycans (Domozych, 2011). The extraction efficiency of compounds from seaweeds is limited due to the presence of these complex cell walls with mixtures of sulphated and branched polysaccharides associated with proteins and various bound ions such as Ca and K (Wijesinghe & Jeon, 2012). Enzymatic-assisted extraction is a relatively recent and environment friendly strategy, which is still scarcely used for the extraction of bioactive compounds from mushrooms; the enzymes hydrolyse cell wall components increasing cell wall permeability resulting in higher extraction yields of solid components (Puri et al., 2012). Enzyme-assisted extraction has shown potential to improve extraction yield, to release secondary plant metabolites and maintain bioactive properties of the extracts (Gil-Chávez et al., 2013). Several studies have reported a higher extractability of bioactive compounds from several brown seaweeds and considered EAE an economic and sustainable method based on solvent-free process with low costs, high extraction rates and high yields (Hardouin et al., 2014; Heo et al., 2003).

Ultrasound-assisted extraction (UAE), as an alternative energy input-assisted extraction method, is an energy input-assisted extraction method that does not require complex instrumentation being relatively low-cost (Dai & Mumper, 2010). It is based on sound waves migration which generates cavitations that promote the release of soluble compounds by disruption of cells and their walls enhancing the mass transfer to the extraction solvent (Cravotto et al., 2008). Ultrasound-assisted extraction and EAE have been reported as alternative approaches with great potential to extract bioactive substances from marine macroalgae (Kadam et al., 2013).

As previously described many extraction techniques are available to extract compounds to be used in different food or nutraceutical applications. Many extraction techniques are indeed available for such purpose yet are not always of favourable application due to associated environmental pollution and costs. Hence, improved extraction techniques are continuously being sought in terms of shortening of operating times, reduction of organic solvent consumption and increase in extraction efficiency. High hydrostatic pressure (HHP) processing has been considered an emerging non-thermal food processing technique that has shown great promise in food and pharmaceutical industries as well as in biotechnological research (Huang et al., 2013). HHP is able to increase the mass transfer rate by changing the solid/liquid ratio gradient and diffusivity, causing damage to the cell membrane and increasing its permeability thus enhancing permeation of the extraction solvent into the cells (Prasad et al., 2012). Since HHP is performed at room temperature it prevents thermal degradation and loss of biological properties of the extracts being prepared (Huang et al., 2013). Several research studies have revealed HHP to be a technique that enables

shorter processing periods, less costs, higher processing safety and increment of extraction yields (Prasad et al., 2012). In fact, according to Huang et al. (2013) there are numerous studies that endorse the use of high pressure as a suitable method to increase the extraction of natural ingredients with added value in terms of nutritional value and biological properties from food and medicinal herb matrices. For example, HHP treatment combined with enzymes increased the extractability and bioactivity of fermented rice bran (Kim & Han, 2012). Higher levels of xanthohumol content in beer wort was obtained by Santos et al. (2013), using HHP treatments opening the possibility to produce healthier beer with higher amounts of xanthohumol, a bioactive compound with anticarcinogenic, antioxidant, anti-inflammatory, and anti-infective activities. In addition, some studies have suggested that HHP processing may enhance the antioxidant activity of strawberry, blackberry, tomato, and carrot purées (Patras et al., 2009a,b). The use of high pressure for extraction of compounds with biological properties from seaweeds can be an alternative way to the conventional extraction methods.

2.5. Functional foods incorporating natural-derived products, extracts and/or ingredients

Natural products and/or their extracts or isolated compounds such as sulphated polysaccharides, chitin or chitosan, proteins and protein hydrolysates, peptides, amino acids such as taurine, omega-3 oils, carotenoids, and other bioactive compounds are examples of ingredients that can be added at different stages, from processing to storage, of the food production process. Natural-derived functional ingredients with biological properties constitute a research area with much to explore for food purposes. On the other hand, many foods already widely consumed all over the world are a potential vector for incorporating new natural-derived functional ingredients, which will enhance their value at both the nutritional and economic levels (Figure 2.4). Since dairy products are widely accepted by the consumers, the use of this type of products to deliver bioactive compounds has received attention in the last years by the food industries (Kadam & Prabhasankar, 2010; Chandini et al., 2008). In several countries of the EU, these types of products are already part of an industry that is already generating millions of Euros/year. Food products containing marine derived chitin, chitosan as well as oils rich in omega-3 fatty acids, are some of the food products that are being commercialized in several markets around the world including Japan, United States, and some European countries (Kadam & Prabhasankar, 2010).

Marine derived functional ingredients are added as fortifiers and nutritional enrichments in food, leading to the so called FF. According to Kadam & Prabhasankar (2010), more and constant efforts in research and design of novel FF based on marine functional ingredients is needed to contribute to the reduction of health problems through the diet. On the other hand, the regular consumer is, in general, afraid to eat less known mushrooms than those available over the year in the market and those are, in general, restricted to few species such as *Agaricus bisporus (champignon)*, *Pleurotus ostreatus (oyster mushroom)*, *Lentinus edodes* (Shiitake), especially in Europe.

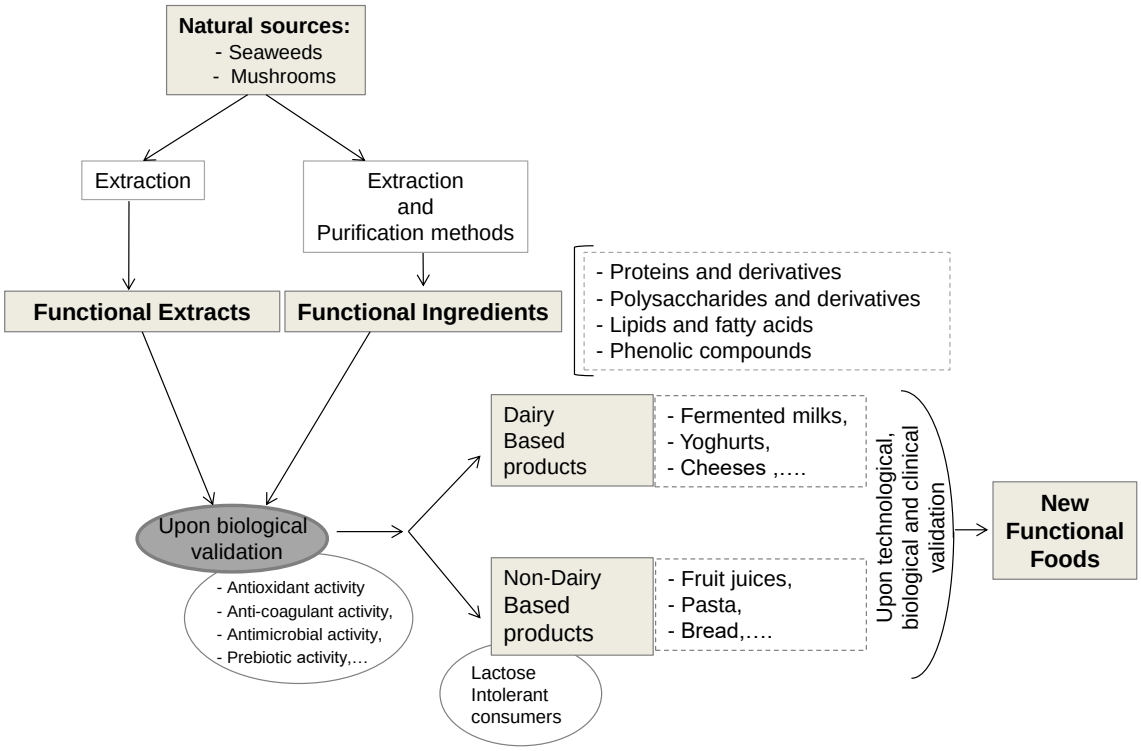

Figure 2.4. New potential functional foods based on dairy or non-dairy products via incorporation of functional extracts and/or ingredients from natural sources such as seaweeds or mushrooms.

Therefore, efforts to design novel FF based on mushrooms functional ingredients would also be helpful to contribute to a more healthy diet (Figure 2.4).

Despite the scientific interest for the use of natural-derived food ingredients there still are various challenges ahead that have to be overcome to design new FF, namely:

i) Efficient extraction methods and purification steps, to obtain food grade validated extracts or purified compounds with biological properties (antioxidant, antibacterial, prebiotic, and others). Isolated functional ingredients should rely upon food compatible methods with economically viable yields. Hence, different extraction methods must be applied in order to maximize the extraction efficiency of functional ingredients with biological properties.

ii) To design FF based on the incorporation of natural-derived functional ingredients upon biological validation; According to Siegrist et al. (2008), consumers are more and more inclined to buy FF with physiological health claims.

iii) Foods should have good sensorial characteristics in order to be accepted by the consumer; In general, consumers are not able to compromise taste for healthiness (Honkanen, 2009). This is in fact one of the most important challenges to be overcome in the case of some of the marine compounds to be used.

2.5.1. Foods incorporating seaweeds or mushrooms

Dietary minerals (Na, K, and I), fibres as well as polyphenolic compounds are some compounds in which seaweeds are rich thus making the use of seaweeds or their extracts to supplement foods of particular interest (Gupta & Abu-Ghannam, 2011) enabling the development of new FF from a nutritional standpoint. The possibility of adding seaweeds to food as natural antioxidant, antimicrobial and texturing agents are other aspects of significant importance from an industrial point of view (Gupta & Abu-Ghannam, 2011). This scenario could perfectly be extrapolated to mushrooms since they are also rich in several compounds with biological interest as described before. In addition, mushrooms ingredients or extracts could surpass or minimize organoleptic impact by seaweeds ingredients or extracts due to their marine-like flavour/aroma which is less tolerable by some consumers.

Meat and meat products which are a good source of protein and vitamins but deficient in fibres and containing, in general, excess Na are good candidates to be supplemented with seaweeds overcoming the technological problem associated with low-salt meat products (Cofrades et al., 2011a; Gupta & Abu-Ghannam, 2011). According to Cofrades et al. (2011a) due to consumer awareness of the negative perceptions of meat in terms of overall fat content, in particular saturated fatty acids, cholesterol and Na, the meat industry should adapt to new meat FF to address consumer concerns without making radical changes in their eating habits. Several studies based on the formulation of meat-based FF incorporating marine compounds have been reported, some of which are described in Table 2.1. In general, all studies presented as main objective the development of low-salt meat products with improved nutritional profile by addition of different species of seaweeds (Cofrades et al., 2011b; López-López et al., 2010, 2009). The major results reported in these studies are encouraging and according to Cofrades et al. (2011b), incorporation of seaweeds allows reformulation strategies to design and develop functional meat products with lower Na, fat, and cholesterol content and simultaneously with relevant contribution to the intake of dietary fibre, polyphenols, minerals and unsaturated fatty acids. Technologically, the incorporation of seaweeds enables overcoming some of the negative sensorial impacts caused by low levels of sodium related to water- and fat-binding properties. In terms of organoleptical properties, some of the developed functional meat products attained sensorial acceptability but this is a subject that should be evaluated in a well based sensorial analysis.

Pasta products incorporating seaweeds have also been developed and two studies are described in Table 2.1. In fact, the design of FF based on incorporation of marine ingredients such as seaweeds, has been more successful in bakery and pasta products and was recently reviewed by Kadam & Prabhasankar (2010). For example incorporation of 10% to 20% edible seaweed wakame (*Undaria pinnatifida*) in pasta not only received sensorial acceptance but it also resulted in a product with improved amino acid and fatty acid profiles, increase of antioxidant activity, and a higher content of fucoxanthin (concentration and activity was not affected by processing) and fucosterol (Prabhasankar et al., 2009). Dietary ingestion of wakame has also been reported to reduce blood pressure (Chandini et al., 2008).

According to Gupta & Abu-Gannam (2011), seaweeds are currently getting considerable consideration for nutritional enrichment of meat, bakery and pasta products because as previously demonstrated the addition of seaweeds or seaweed extracts can not only improve the quality of the product but may even enhance their safety at high doses concerning natural antioxidants and antimicrobials.

In terms of mushrooms, Ulziijargal et al. (2013) studied the incorporation of different mushrooms mycelia in bread as a substitute 5% of wheat flour. Incorporating 5% mushroom mycelia into the bread formula did not adversely affect the texture profile of the bread and after baking, mycelium-supplemented bread still contained substantial amounts of χ-aminobutyric acid and ergothioneine (0.23–0.86 and 0.79–2.10 mg/g dry matter, respectively) however, lower bread' s acceptability was noted. Mushroom powder has also been incorporated in other bakery products with compatible quality to wheat flour counterparts. A healthy version of muffins and cookies were produced with *Lenthinus edodes* and *Pleurotus eryngii* powders respectively (Kim & Joo, 2012; Kim et al., 2010).

Mushrooms have also been incorporated in pork patties as a functional ingredient. The texture, juiciness and functionality of pork patties was improved with the use of *Lenthinus edodes* powder (Chun et al., 2005). On the other hand, white jelly mushroom (*Tremella fuciformis*) was used to increase the oil-holding capacity of pork patties, maintaining a good sensory evaluation score and simultaneously lowering energy content (Cha et al., 2013). The *Agaricus brasiliensis* mushroom was successfully used in the development of a burger-type product. The developed was well accepted by consumers and a 12% mushroom content led to higher nutritional value compared to the bovine counterpart, presenting high protein content (20.31%), carbohydrates (27.84%), dietary fibre (24.47%) and ash (6.12%) (Lemos, 2009).

Albeit the addition of mushrooms directly in foods mushrooms have also been studied as natural seasonings. For example, *Pleurothus ostreatus* and *L. edodes* have a savoury flavour given their nucleotide content. *Lenthinus edodes* has been successfully used as a flavouring agent in brown sauce with added value given its antioxidant activity (Yoo et al., 2007). Furthermore, the addition of *A. brasiliensis* to tomato sauces increased the polyphenol content in comparison to other control tomato sauces. Its contents in glucan complex, b-carotene and lycopene led to tomato sauces rich in b-glucan, carotenoids and lycopenes (Miller et al., 2005). Functional beverages have also been a target for adding mushrooms. For example, *Ganoderma lucidum* powder was used as 0.1% in the production of Yakju, a Korean rice wine. Results revealed a beverage with a very positive sensory score and with antihypertensive properties (Kim et al., 2004).

Table 2.1. Functional foods based on incorporation of seaweeds

	Product	Seaweed	Description	Major Achievements	Sensory Characteristics	Ref.
Meat	Frankfurters sausages	*Himanthalia elongata*	Low-fat frankfurters added with 5.5% seaweed	- Addition of seaweed provided a Ca-rich, low-Na sausages, better Na/K ratios and higher content in fiber	- No sensory analysis presented	Lopéz-Lopéz, 2009
			Low-fat, low-salt frankfurters added with konjac glucomannan gel (0 to 19.3%) and seaweed (o to 3.3%)	- Incorporation of combination of konjac gel and seaweed increased cooking loss and reduced emulsion stability - Replacement of pork fat, reducing fat content in 15%, did not impart noticeable changes in the sensory quality of the frankfurters	- No significant differences among samples in terms of texture - No noticeable changes in sensory quality as a consequence of fat reduction and fat replacement - Samples with added seaweed with low score in terms of overall acceptability	Jimenez-Colmenero et al., 2010
	Beef Patties	*Undaria pinnatifida*	Low-salt and low fat beef patties added with 3.3% of seaweed and partial fat replacement with olive oil	-Patties with seaweed presented lower thawing and cooking losses and were softer - Seaweeds incorporation increased mineral contents but impart similar Na/K ratio - Good technological, sensorial and nutritional properties in beef patties with seaweed and olive oil emulsion	- Frozen did not affected sensorial studied parameters - Total replacement of fat by olive oil emulsion improved appearance - Seaweed incorporation did affect the sensory properties	Lopéz-Lopéz, 2010
	Restructured Poultry Steaks	*Himanthalia elongata*	Low-salt restructured poultry with microbial transglutaminase and seaweed (3%)	- Addition of a cold binding agent (MTGase/caseinate) did not affect water binding properties - Incorporation of seaweed caused a slight increase in purge loss but reduced cooking loss - Addition of both, seaweed and binding agent, increased the Kramer shear force	- Addition of seaweed and MTGase/caseinate significantly affected most sensorial parameters - Flavor acceptability, juiciness and general acceptability scored with values higher than 5 - Low-salt restructured products with seaweed and MTGase/caseinate were considered sensorial acceptable	Cofrades et al., 2011b
	Pork Patties	*Saccharina japonica formely Laminaria japonica*	Reduced-fat pork patties with added seaweed (0-5%)	- Reduced-fat pork patties with seaweed had significantly higher moisture, ash, carbohydrate content and lower protein and fat content, cooking loss, hardness than regular-fat control samples	- Pork patties with fat content reduced to10% and supplemented with 1-3% of seaweed has improved quality characteristics, similar to control patties with 20% fat content.	Choi et al., 2012
Pasta	Fresh Noodles	*Monostroma nitidum*	Noodles added with 4 to 8% of seaweed powder with or without eggs	- Addition of seaweeds increased crude fiber content, higher cooking yields and decreased springiness and extensibility	- Sensory scores varied with the parameters evaluated, percentage of seaweed and addition of eggs; - Higher scores for color and wetness were obtained in egg noodles with 8% of seaweed	Chang et al., 2008
	Pasta	*Undaria pinnatifida*	Pasta prepared with semolina and seaweed blends: 100:0, 95:5, 90:10, 80:20 and 70:30	- Fucosterol and fucoxanthin content higher in pasta with seaweeds, which were not affected by cooking step - Improved nutritional, amino acid and fatty acid profiles by incorporation of seaweed.	- Pasta with 10% seaweed was considered acceptable sensory-wise with mild seaweed flavor tasting similar to control pasta	Prabhasankar et al., 2009

2.5.2. Foods incorporating natural-derived ingredients

Complex marine-derived polysaccharides depending on the chemical composition and structure can possess several biological properties, as previously described. Therefore, their incorporation in food matrices is of potential interest to develop new FF. In Table 2.2, are listed some studies based on incorporation of marine-derived polysaccharides as functional ingredients such as alginates and carrageenan in bread, meat or liquid foods. New types of healthier breads, especially gluten-free bread, a rising market due to the increasing diagnostic of celiac disease among adult individuals (Houben et al., 2012), has been a challenge to bakers and scientists. Agar-agar and carrageenan are two hydrocolloids that can be used as thickening, swelling, stabilizing or humectants agents which can be used in gluten-free baking (Houben et al., 2012). Different types of bread with incorporation of sodium alginate and carrageenan was attempted and investigated in terms of baking properties (Mikuš et al., 2013; Sim et al., 2011; Guarda et al., 2004). In general, marine derived polysaccharides were able to impart acceptable textural and sensorial characteristics. However, none of these studies carried out nutritional evaluation which could enhance some healthy aspects due to the incorporation of marine-derived polysaccharides. Meat sausages as well as milk and apple juice were other food matrices tested with supplementation of carrageenan, chitosans and chito-oligosaccharides (Table 2.2). Satisfactory technological and sensorial results were achieved by Ayadi et al. (2009) but again no nutritional evaluation was performed. Fernandes et al. (2008) studied the incorporation of commercial crab shells chitosans and chito-oligosaccharides in apple juice and milk to ascertain the influence of food components on their antimicrobial activity, evaluating in parallel the acceptance by a sensory panel. Once again no nutritional evaluation was considered. In Japan, several foods (potato chips, soybean paste, and noodles) with added chitosan are available as cholesterol-lowering FF (Borderías et al., 2005).

Results reported by Fernandes et al. (2008) are an example of the difficulties that need to be overcome in the development of new FF. The antibacterial effect against pathogenic *Staphylococus aureus* and *Escherichia coli* was dependent on bacteria Gram nature and chitosans' molecular weight, yet their addition to apple juice led to some unpleasant off-flavours which steadily increased in intensity with chitosan molecular weight (Table 2.2). In fact, the incorporation of marine ingredients in foods of different nature could lead the consumer to expect a negative influence on food (Honkanen, 2009). The importance of the food vector has been shown in many studies and indeed a high correlation between food vector and functional ingredient origin is required in order to promote functional food acceptance (Siegrist et al., 2008).

Table 2.2. Functional foods based on incorporation of marine-derived polysaccharides

Product		Marine-derived Polysaccharide	Description	Major Achievements	Sensory Characteristics	Ref.
Bread	Bread	Sodium alginate k-carrageenan	Bread produced with different hydrocolloids at 0.1-0.5% (flour basis)	- All hydrocolloids were able to reduce loss of moisture content during bread storage, reducing dehydration rate of crumb - Higher moisture content and specific volume index in bread with k-carragenaan than with sodium alginate - Alginate showed anti-staling effect retarding the crumb hardening - No nutritional evaluation	- Bread with 0.5% of alginate were scored higher in terms of visual appearance, crunchiness and overall acceptability than control bread or bread with 0.1 to 0.5% of k-carragenan	Chang et al., 2008
	Wheat dough, Chinese steamed Bread	Sodium alginate	Wheat dough Chinese steamed bread added with sodium alginate (0.2% w/w) and konjac glucomannan (0.8% w/w)	- Addition of alginate sodium and konjac glucomannan produced dough with rigid and weak network - Lower spread ratio and specific volume Chinese steamed bread with addition of alginate sodium and konjac glucomannan but softer and more resistant to staling through storage than control samples - No nutritional evaluation	- No sensory analysis presented	Prabhasankar et al., 2009
	Wheat Bread	Carrageenan	Wheat bread added with enzymes and hydrocolloids	- Lower volume and specific volume was observed in bread with carragenan (0.075% flour basis) but higher when conjugated with xanthan gum (0.15%, flour basis) than in standard bread - Lower water losses in bread with carragenan alone or mixed with xanthan gum than in standard bread - No nutritional evaluation	- Higher scores for sensorial properties (taste, color, shape, aroma, elasticity, hardness) in bread with carragenan, especially when mixed with xanthan gum in comparison to standard bread	Guarda et al., 2004
Meat	Sausages	Carrageenan	Turkey meat sausages with addition of carrageenan (0-1.5%, w/w)	- Carrageenan causes a decrease in emulsion stability but an increase in water holding capacity, hardness, and cohesiveness - Addition of 0.2 to 0.5% of carrageenan increases gel elasticity; higher % of carrageenan cause a opposite effect - Higher % of carrageenan lead to a progressive appearance of an additional carrageenan gel network - No nutritional evaluation	- Addition of carrageenan did not cause significant effect of sausage taste but improved sausage appearance and texture	Sim et al., 2011
Beverage	Milk Fruit Juice	Chitosans Chitooligosacharides (COS)	Cow milk and apple juice added with 0.5% (w/v) of chitosan with low to high molecular weight (MW) and COS lower than 3 and 5 Kda	- Antimicrobial effectiveness against to *Escherichia coli* and *Staphylococcus aureus* by medium and high Mw chitosans when incorporated in milk and apple juice, COS lost their antimicrobial activity upon both bacteria after 4-8h in milk - Chitosans and oligomers are more effective as food preservatives in low pH foods and with low protein content - No nutritional evaluation	- Addition of chitosans to apple juice cause unpleasant off-flavors: astringency and after taste especially for higher MW - No sensorial analysis was performed on milk added with chitosans and COS because milk become unsuitable for consumption	Mikuš et al., 2013

Cereal-based products like oat and soy bars and spaghetti seem to be the more suitable food products to deliver long-chain omega-3 PUFAs to the consumers (Hughes et al., 2012; Iafelice et al., 2008) with negligible levels of oxidation. According to Kadam & Prabhasankar (2010), bakery and pasta products are the best food products to incorporate marine functional ingredients being widely consumed all over the world.

In terms of mushrooms functional ingredients, Mesomo et al. (2010) determined the chemical composition of the *Agaricus blazei* residue obtained after β-glucans aqueous extraction and the shelf-life of cheese bread made with this product. The results of sensory analysis showed that *A. blazei* Murrill residue is an excellent source of nutrients and its addition to the cheese bread formulation was favourable in all evaluated attributes; the sample with the higher storage time had good acceptance, which shows that it is possible to store the product for approximately 30 days without great flavour, texture and appearance negative changes.

2.6 Current understanding and final considerations

Although there are already commercially available marine-derived ingredients and FF, the search for new natural-derived products continues to be a challenge both for scientists and food engineers. The search and use of marine-derived or mushroom functional ingredients demands appropriate and sustainable harvesting and that extraction of compounds with bioactive potential be based on food grade compatible, rapid, efficient and sustainable proceedings followed by procedures to obtain concentrated extracts or purified compounds in a controlled manner. The validation of biological properties of extracts and/or purified functional marine-derived or mushroom-derived ingredients is determinant for the development of new FF based on their incorporation in food matrices which in turn should be followed by characterisation thereof and assessment of stability and *in vitro* bioavailability of functional ingredients; proof of the specific health benefits through human clinical trials is also an important requirement.

Natural resources such as seaweed and mushrooms can be used to obtain new products, providing an alternative and sustainable manner to provide new FF or ingredients with biological properties that may help to develop new strategies based on preventive health. The use of natural resources can contribute to economic improvement through job creation and reduction of potential costs related to health. As consumers worldwide become more health conscious, the demand for health-promoting foods and food components is expected to grow. The market for such foods is predicted to be quite large. Before the full market potential can be realized, however, consumers will need to be assured of the safety and efficacy of FF. Current and future scientific studies are needed to provide this assurance and to inspire confidence in FF in the minds of consumers worldwide, particularly those derived from seaweeds and mushrooms ingredients.

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Chapter 3

Chemical composition and nutritive value of a selection of edible seaweeds from Buarcos bay in Central West Coast of Portugal

3.1. Introduction

Algae, in particular, edible seaweeds, are a very interesting natural source of compounds with biological activity that could be used as functional ingredients as previously described in chapter 2. Seaweeds can in fact provide several compounds at different quantities and for that, research on identification of bioactive compounds from algae can be seen as an almost unlimited field (Lordan et al., 2011). According to Chojnacka et al. (2012), there are about ten thousand identified algae species, yet only about 5% thereof are used as human food or as animal feed. More than one hundred seaweed species are used worldwide, especially in Asian countries, where they are used as sea vegetables. In Southern Europe the use of edible seaweeds for food purposes is still residual, not yet fitting with the regular consumption habits. However, this trend is changing and currently researchers are searching for, and studying, less-known seaweed species not only because of their potential biological properties due to specific functional compounds but mostly for their nutrient profile, which is being considered as important alternative protein and complex carbohydrate sources (Ibañez & Cifuentes, 2013).

The main objective of the study described in this chapter was to determine the chemical composition providing the details in nutritional properties of six different edible species from each of the main seaweed groups (red, brown and green algae) occurring at Buarcos Bay (Figueira da Foz, Portugal). The selection was based on representative species from the Central West Portuguese Coast some of which have inclusively very few studies on its properties, including the less studied *Osmundea pinnatifida*. New information on the proximal composition, fatty acids profile and elemental composition of the six seaweeds is presented as well as the identification of the principal seaweed colloids by FTIR-ATR and FT-Raman.

3.2. Material and methods

3.2.1. Specimens of seaweeds

Specimens of red algae (Rhodophyta, Florideophyceae) *Osmundea pinnatifida* (Ceramiales), *Grateloupia turuturu* (Halymeniales) and *Gracilaria gracilis* (Gracilariales), brown algae (Heterokontophyta, Phaeophyceae) *Sargassum muticum* (Fucales) and *Saccorhiza polyschides* (Tilopteridales) and of green algae (Chlorophyta, Ulvophyceae) *Codium tomentosum* (Bryopsidales), were harvested in April 2012 from Buarcos bay (Figueira da Foz, Portugal). The classification of these seaweeds was based on *AlgaeBase* (Guiry & Guiry, 2013). All six selected species are edible being used as food for humans all around the world (SIA, 2015; Munier et al., 2013; Lodeiro et al., 2012; Roo et al., 2007). The seaweeds were first washed with running tap water and then with deionised water to eliminate residues from the thalli surface and then dried in an oven at 60 ºC. The dried seaweeds were milled to less than 1.0 mm particle size.

3.2.2. Chemical characterization of seaweeds

3.2.2.1. Proximate composition

Content in moisture, organic matter and ash were determined according to AOAC methods (1990). Nitrogen content was determined by the Kjeldahl method adapted from where protein content is estimated by multiplying the nitrogen content by 6.25 (Munier et al., 2013; Denis et al., 2010) whereas total fat content was determined by Soxhlet extraction. Total sugar content was determined by calculation, i.e. by subtracting protein content and fat content from total organic content. Total polyphenols were extracted from 0.1 g of dry seaweed in 10 mL of ethyl acetate after 30 min of sonication (on a water bath ultrasonicator, Ultrasonik 57H Ney). The extract was filtered with anhydrous sodium sulphate (Sigma) and brought to dryness with a rotary evaporator (Laborato 4000, Heidolph). The residue was re-dissolved in 5 ml of milliQ water and the content of total polyphenols of 2 mL was determined by colorimetric method of Folin-Ciocalteu, using cathecol (0 to 75 mg/L) as standard and expressed as μg cathecol equivalent per g of dry seaweed.

3.2.2.2. Analysis of fatty acids

Hexane and methanol were HPLC grade (VWR Scientific, West Chester, PA). Sodium sulphate was analytic grade and purchased to Panreac (Barcelona, Spain). Methyl tricosanoate (99%) and Supelco 37 FAME mix were obtained from Sigma (Sigma, USA). GLC-Nestlé36 was purchased to Nu-Chek Prep, inc. (Elysian, Minnesota, USA) while butterfat CRM-164 (EU Commission; Brussels, Belgium) was from Fedelco Inc. (Madrid, Spain).

For the analysis of the total fatty acid (FA) composition, 100 mg of sample were accurately weighed and prepared according to Sánchez-Avila et al. (2009). For quantification purposes samples were added with 100 µL of methyl tricosanoate (1.28 mg/mL) prior to derivatization. FAME were analysed in a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (50m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France) according to the conditions described by Vingering & Ledoux (2009). Supelco 37 and CRM-164 were used for identification of fatty acids. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.15 µg/mL; LOQ: 0.46 µg/mL).

3.2.2.3. Elemental composition

3.2.2.3.1. Microwave-assisted acid digestion procedure

The microwave-assisted digestion proposed by Speedwave MW-3+ (Berghof, Germany) for dried plant samples with some modifications was used for determination of Mo, B, Zn, P, Cd, Co, Ni, Mn, Fe, Mg, Ca, Cu, Na, Al and K in dried seaweed samples. A sample with up to 0.2 g dry seaweed was placed in the digestion vessel and added with 5 mL of concentrated nitric acid. The vessels were capped and placed in a microwave pressure digestor Speedwave MWS-3+ (Berghof) and subjected to microwave radiation at 20 bar according to the following program: room temperature was raised first to 130 ºC at 22 ºC/min and 30% of irradiation power, then to 160 ºC at 6 ºC/min and 40% of irradiation power, remaining 5 min at this temperature, and to 170 ºC at 5 ºC/min and 50% of irradiation power, remaining 5 min at this temperature. The cooling process consisted in decreasing temperature first to 100 ºC for 4 min and then to room temperature. After cooling, acid digests were made up to 20 mL with Milli-Q water. Three replicates were performed for each seaweed sample as well as blanks. The content of each element is expressed as the mean plus standard deviation

3.2.2.3.2 Determination of 15 elements

The elemental composition was determined using an inductively coupled plasma (ICP) optical emission spectrometer model Optima™ 7000 DV ICP-OES (Dual View, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) with radial plasma configuration.

Standard plasma conditions were used namely 1300 W for radio-frequency power, 1.5 mL/min pump rate, and 15.0, 0.2 and 0.8 L/min for plasma, auxiliary and nebulizer gas flow, respectively. Detection wavelengths were 202.031, 208.889, 213.857, 214.914, 226.502, 228.616, 231.604, 257.610, 259.939, 279.955, 317.933, 324.752, 330.237, 394.401, 769.896 nm for Mo, B, Zn, P, Cd, Co, Ni, Mn, Fe, Mg, Ca, Cu, Na, Al and K, respectively.

A multi-element standard (Inorganic Ventures) containing up to 10 mg/L of Mo, 2.5 mg/L of B, 15 mg/L of Zn, 750 mg/L of P, 0.2 mg/L of Co and Ni, 1 mg/L of Cd and Cu, 15 mg/L of Mn and Fe, 1000 mg/L of Mg, 3000 mg/L of Ca, 50 mg/L of Na, 5 mg/L of Al and 2000 mg/L of K was used for the preparation of standard solutions in 2% HNO_3. Successive dilutions of the stock reference solution (100, 50 and 10 times) were prepared and used for calibration models and the concentration of each element was determined by direct interpolation in the standard curve within its linear dynamic range. The limits of detection (LODs) were calculated using y=yB+3SB, where SB is the standard deviation (SD) of the blank signal estimated as sy/x, the residual SD taken from the calibration line, and yB is the blank signal estimated from the intercept, also taken from the calibration line. The LODs were found to be 0.010, 0.0009, 0.010, 0.40, 0.002, 0.002, 0.003, 0.014, 0.16, 0.18, 2.06, 3.32, 1.17, 0.037, 3.75 mg/L for Mo, B, Zn, P, Cd, Co, Ni, Mn, Fe, Mg, Ca, Cu, Na, Al and K, respectively. Acid digests for some seaweed samples were diluted up 400 times to assess the content of some elements in particular for Mg, Na, K and Fe within calibration curve range values.

The accuracy of the method (microwave acid digestion and ICP-OES analysis) was assessed by analysis of certified reference material NIES-03 (Seaweed Chlorella; LGC standards, UK). Five replicates of reference material were subject to microwave digestion and analysed three times by ICP-OES. Recovery ranged between 89 and 108 %.

3.2.2.4. FTIR-ATR and FT-Raman Analysis

Samples of milled, dried algal material were analysed by Fourier Transform Infrared Spectroscopy with attenuated total reflectance (FTIR-ATR) and Fourier Transform Raman spectroscopy (FT-Raman) according to the method described by Pereira (2006) and by Pereira et al. (2009).

The FTIR spectra of milled dried seaweed were recorded on an Bruker Tensor 27 spectrometer (Bruker Scientific Instruments, Massachusetts, USA), using a Golden Gate single reflection diamond ATR system (Specac Lda, Cranston, USA), with no need for sample preparation. All spectra resulted from the average of two counts, with 128 scans each and a resolution of 2 cm^{-1}.

The room temperature FT-Raman spectra were recorded on a RFS-100 Bruker FT-spectrometer using a Nd:YAG laser with excitation wavelength of 1064 nm. Each spectrum was the average of two repeated measurements, with 150 scans at a resolution of 4 cm^{-1}. For FT-Raman, the samples of seaweeds were first submitted to depigmentation by immersion in cold calcium hypochlorite (4%) for 30-60 s at 4 ºC and then washed and dried at 60 ºC.

3.2.3. Statistical analysis

One-way analysis of variance (ANOVA) was carried out with SigmaStat™ (Systat Software, Chicago, IL, USA), to assess differences between seaweed species in terms of proximate or elemental composition at a significance level of p=0.05. Normality and homogeneity were examined and the Holm-Sidak method was used for pairwise comparisons (p<0.05). FA data in turn, were analysed using the IBM SPSS Statistics v22 for Mac. Normality and homogeneity were examined and one-way ANOVA with the Bonferroni test for post-hoc analyses was applied to evaluate statistical differences between seaweeds (p<0.05).

3.3. Results and discussion

3.3.1. Proximate composition of seaweeds

The proximate composition of the different edible seaweeds' species is displayed in Table 3.1. Seaweeds are perishable and deteriorate rapidly within a few days upon harvesting and therefore drying is an essential step to preserve them. Drying decreases the water activity which ultimately

retards microbial growth, helps to preserve the desirable qualities and reduces storage volume. In general, moisture content ranged between 8.0 g/100$g_{dry\ seaweed}$ in dried *G. gracilis* to 11.8 g/100$g_{dry\ seaweed}$ in dried *O. pinnatifida*; the moisture content was statistically different (p<0.05) among all species tested except between *O. pinnatifida* and *G. turuturu*.

The protein content, one of the major biochemical components of seaweeds, ranged from 14.4 g/100$g_{dry\ seaweed}$ in *S. polyschides* (brown seaweed) to 23.8 g/100$g_{dry\ seaweed}$ in *O. pinnatifida* (red seaweed) being the protein content statistically different (p<0.05) between all seaweed species. Highest values of protein content were found in the red seaweed species (20.2 to 23.8 g/100$g_{dry\ seaweed}$), followed closely by the green seaweed *C. tomentosum* (18.8 g/100$g_{dry\ seaweed}$) and with lowest content were the brown seaweed (14.4 to 16.9 g/100$g_{dry\ seaweed}$) (Table 3.1). According to Ibañez & Cifuentes (2013), protein content differs between seaweed species and, in general, red (Rhodophyta) and green (Chlorophyta) seaweed species are characterized by higher protein content in comparison to brown seaweed species (Phaeophyceae). Similar values were described by Denis et al. (2010) for *G. turuturu* collected in Brittany (France) (23 % of protein content) and by Paiva et al. (2014) for *O. pinnatifida* collected in Azores Archipelago (21% of protein content), respectively. However higher values (27%) are reported for the same species collected in North Yorkshire (UK) by Marsham et al. (2007). Patarra et al. (2011) reported 23% of total protein in *G. turuturu* collected in the Azores Archipelago coast during mid-winter period, which is very similar to that reported herein for *G. turuturu* collected in the Spring, both periods during which algal protein content is at its highest level. Jard et al. (2013) reported 84 to 120 g/K$g_{dry\ seaweed}$ of protein content for *S. muticum*, *S. polyschides* and *C. tomentosum* collected in French Brittany. Protein contents found in the red and green seaweeds analysed are similar to the protein contents of many legumes such as peas or beans (19-22%) or meats (18-25%). Therefore, these seaweeds from the Central Portuguese coast may very well be used in the formulation of low-cost, protein balanced diets that may alternate with current vegetable protein sources such as legumes and cereals.

Table 3.1. Proximate composition of seaweeds and their percentage contribution in nutrients intake.

Parameter	*G. gracilis*	*O. pinnatifida*	*G. turuturu*	*S. muticum*	*S. polyschides*	*C. tomentosum*
% Moisture ($g/100g_{dry\ seaweed}$)	7.99±0.02 a	11.77±0.01 e	11.68±0.05 e	9.64±0.08 c	10.88±0.04 d	9.0±0.2 b
% Total Protein ($g/100g_{dry\ seaweed}$)	20.2±0.6 d	23.8±0.6 f	22.5±0.3 e	16.9±0.2 b	14.44±0.1 a	18.8±0.1 c
% Total sugars[1] ($g/100g_{dry\ seaweed}$)	46.6	32.4	43.2	49.3	45.6	32.8
% Total Fat ($g/100g_{dry\ seaweed}$)	0.60±0.01 a	0.9±0.1 a	2.2±0.1 c	1.45±0.07 b	1.1±0.1 ab	3.6±0.2 d
Total phenolic content (μg cathecol equiv/$g_{dry\ seaweed}$)	228±14 a	337±22 b	208±8 a	499±32 c	224±13 a	920±84 d
% Organic matter ($g/100g_{dry\ seaweed}$)	67.21±0.01 d	57.6±0.2 a	67.80±0.06 d	67.41±0.02 d	60.97±0.05 c	55.0±0.7 b
% Ash ($g/100g_{dry\ seaweed}$)	24.8±0.03 c	30.62±0.25 e	20.52±0.01 a	22.94±0.06 b	28.15±0.01 d	35.99±0.48 f

		G. gracilis		*O. pinnatifida*		*G. turuturu*		*S. muticum*		*S. polyschides*		*C. tomentosum*	
Nutrients	g/day	$g/10g_{portion}$	%RDI[3]	$g/10g_{portion}$	%RDI[3]	$g/10g_{portion}$	%RDI[3]	$g/10g_{portion}$	%RDI[3]	$g/10g_{portion}$	%RDI[3]	$g/10g_{portion}$	%RDI[3]
Protein	50	2.0	4.0	2.4	4.8	2.2	4.5	1.7	3.4	1.4	2.9	1.9	3.8
Total sugars	270	4.7	1.7	3.2	1.2	4.3	1.6	4.9	1.8	4.6	1.7	3.3	1.2
Fat	70	0.06	0.1	0.09	0.1	0.22	0.3	0.15	0.2	0.11	0.11	0.36	0.5
	mg/day[2]	$mg/10g_{portion}$	%RDI[3]	$mg/10g_{portion}$	%RDI[3]	$mg/10g_{portion}$	%RDI[3]	$mg/10g_{portion}$	%RDI[3]	$mg/10g_{portion}$	%RDI[3]	$mg/10g_{portion}$	%RDI[3]
Calcium	800	34.4	4.3	54.1	6.8	26.5	3.3	91.8	11.5	91.1	11.4	51.3	6.4
Potassium	2000	651.0	32.6	261.0	13.0	162.8	8.1	575.6	28.8	765.4	38.3	372.9	18.6
Magnesium	375	17.5	4.7	48.0	12.8	69.5	18.5	150.4	40.1	79.7	21.3	104.6	27.9
Phosphorus	700	22.6	3.2	17.3	2.5	28.1	4.0	22.8	3.3	23.2	3.3	18.0	2.6
Iron	14	0.90	6.5	3.7	26.2	0.50	3.6	1.9	13.4	0.79	5.7	2.83	20.2
Zinc	10	0.25	2.5	0.58	5.8	0.69	6.9	0.25	2.5	0.65	6.5	0.18	1.8
Copper	1	0.04	4.5	0.05	5.0	0.03	3.3	0.05	4.5	0.03	3.4	0.06	6.3
Manganese	2	0.20	9.9	0.12	5.8	0.25	12.6	0.11	5.4	0.08	3.8	0.19	9.4

[1]% Total Sugars (%) = Organic matter (%) – Total Protein (%) - Total Fat (%); a-f, in a row: Different letters indicate significant differences ($p<0.05$) between species;
[2]Data from Mišurcová et al. (2011); [3]Based on daily intake portion of 10 g of dry seaweed.

Seaweeds are also known for their low fat content which varies significantly throughout the year according to Manivannan et al. (2008). A range between 0.6 to 3.6 g/100$g_{dry\ seaweed}$ of total fat was observed in the studied seaweeds (Table 3.1). The lowest fat contents were reported for the red seaweeds *O. pinnatifida* and *G. gracilaris* which differed significantly from the highest fat content reported for the green seaweed *C. tomentosum*; no significant differences were found between the fat content of the red and brown seaweed classes. The analysed fat contents did not always agree with those of previous studies: lower contents in *O. pinnatifida* (0.9 g/100$g_{dry\ seaweed}$) were found than values of 4.3% and 7.5% fat reported by Marsham et al. (2007) and by Paiva et al. (2014), respectively, whereas higher values of fat content were observed in *C. tomentosum* (3.6 g/100$g_{dry\ seaweed}$) than the 2.5% fat content reported by Manivannan et al. (2008). In the case of *S. muticum* analysed fat content values were comparable to those found for *S. muticum* collected in French Brittany (19 g/$kg_{dried\ seaweed}$) and reported by Jard et al. (2013). As previously mentioned, sampling period and location may account for such differences. Munier et al. (2013) studied the influence of two sampling sites located in the intertidal zone of Atlantic Coast (Le Croisic and Batz-sur-mer) in Brittany (France) on the biochemical composition of *G. turuturu.* This study demonstrated that the sampling site influenced the biochemical content (protein, lipid and water-soluble carbohydrates) of *G. turuturu;* higher contents of total protein content, lipid and total water-soluble carbohydrates were observed in specimens collected in Le Croisic which had thicker thalli and were less viscous and more greenish than those collected in Batz-sur-mer. According to authors the morphology of thalli could be related to environmental characteristics found in each sampling location.

The total sugar content ranged from 32.4 g/100$g_{dry\ seaweed}$ in *O. pinnatifida* (red seaweed) to 49.3 g/100$g_{dry\ seaweed}$ in *S. muticum* (brown seaweed) (Table 3.1) being of similar order of magnitude to published values by Denis et al. (2010) for *G. turuturu* (43 to 49%) but higher than those reported by Jard et al. (2013) for S. *polyschides*, *S. muticum* and *C. tomentosum* (159 to 176 g/$kg_{dry\ seaweed}$) and by Paiva et al. (2014) for *O. pinnatifida*. Once again, greater variability in total sugar content was observed among red seaweed than among brown seaweed.

Most of the polyphenols, including phenolic acids or polyphenolic compounds, isolated from marine sources are of macro and micro-algae origin and they have been associated with antioxidant properties; in general, lower degrees of polymerization result in greater antioxidant properties. In this study, the highest total phenolic content was observed in the green seaweed *C. tomentosum* (920 μg cathecol equiv/$g_{dry\ seaweed}$) followed by brown seaweed *S. muticum* (499 μg cathecol equiv/$g_{dry\ seaweed}$) and by the red seaweed *O. pinnatifida* (337 μg cathecol equiv/$g_{dry\ seaweed}$). The phenolic content was statistically different (p<0.05) among all species tested except between *G. gracilis*, *G. turuturu* and *S. polyschides.*

Tanniou et al. (2014) studied the variability of phenolic contents of the invasive brown seaweed *S. muticum* collected in several countries along European Atlantic coast from Southern Portugal to south coast of Norway. Interestingly, higher phenolic content was reported in *S.*

muticum (2.46±016 to 4.28±0.26% dry weight seaweed) collected in Portugal in comparison to those collected in Norway, Ireland, France or Spain.

Ash percentage was also quite variable among the different species tested ranging from 20.5 g/100$g_{dry\ seaweed}$ in *G. turuturu* to 36 g/100$g_{dry\ seaweed}$ in *C. tomentosum* (Table 3.1). The differences between the studied seaweed species were all statistically significant (p<0.05) even between red or brown seaweeds. The observed values for ash content are in accordance to published values for *O. pinnatifida* (32.3%) and for *G. turuturu* (18.5%) by Denis et al. (2010). It is known that higher levels of ash are associated with higher amounts of mineral elements.

3.3.2. Fatty acids composition of seaweed

Although quantitatively low, the qualitative profile of FA composing the total fat content is of particular interest from a nutritional point of view. Indeed, analysed seaweeds revealed an important, yet complex, fatty acid profile (Table 3.2). In general, these seaweeds were mainly composed of saturated (SFA) and polyunsaturated FA (PUFA), which ranged from the branched chain C13ai to C22:5 n3 (Docosapentaenoic acid, DPA). Palmitic acid (C16) was the main SFA compound in all samples. This FA reached values of 52.54 g/100g_{fat} in *G. gracilis* while the lowest amount was found in *S. polyschides* (25.49 g/100g_{fat}; p<0.05). However, this latter species together with *C. tomentosum* and *G. turuturu* had the highest total FA contents (19.96, 27.58 and 20.89 µg/$g_{dry\ seaweed}$ respectively, p<0.05). In all samples except for *O. pinnatifida*, C16 Phy (Phytanic acid) was the third most important SFA after myristic acid (C14). The maximum level of C16 Phy was for *S. polyschides* (3.85 g/100g_{fat}). The presence of this compound is interesting since it has been elsewhere suggested to have preventive effects on metabolic dysfunctions due to retinoid X receptor (RXR) and peroxisome proliferator-activated receptor-alpha (PPAR-alpha) agonist activity (Hellgren, 2010).

The content of omega-3 (n3) and omega-6 (n6) varied among the studied seaweed species. The n3 FA accounted for 1.3 to 31.5%, n6 FA for 6.7 to 27.4% whereas n9 FA accounted for 6.6 to 13.5% (Table 3.2). C20:5 n3 (EPA) was for some seaweed species the most characteristic PUFA showing 29.92 g/100g_{fat} in *G. turuturu* or 15.58 g/100g_{fat} in *O. pinnatifida.* These FA have gained much attention since they are related to the prevention of cardiovascular (CVD) and coronary diseases (CHD). The current guidelines recommend an intake of 0.25-2 g of combined eicosapentaenoic acid (C20:5n-3) and DPA (C22:5n-3) (WHO, 2008) but those doses cannot be fully obtained from the analysed seaweeds. Furthermore, the observed contents of arachidonic acid (AA; 18.62 g/100g_{fat} for *G. gracilis* to 2.86 g/100g_{fat} for *C. tomentosum*) and its association with inflammatory processes open the need to study their possible effects on human metabolism. In addition, *S. muticum* and *C. tomentosum* also showed high amounts of γ-linolenic acid (C18:3 c6 c9 c12; 8.87g/100g_{fat} and 2.84 g/100g_{fat} respectively).

Table 3.2. Fatty acid composition (g FA/100g_{fat}) and total content (µg FA/$mg_{dry\ seaweed}$) of the seaweeds.

	G. gracilis		*O. pinnatifida*		*G. turuturu*		*S. muticum*		*S. polyschides*		*C. tomentosum*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C13ai	0.30 c	0.09	0.48 c	0.06	0.37 c	0.07	1.05 a	0.09	0.71 b	0.03	0.40 c	0.07
C14	5.57 b	0.01	6.46 a	0.01	2.90 d	0.02	2.94 d	0.01	3.85 c	0.03	1.01 e	0.32
C14:1 c9	0.87 a	0.01	0.08 c	0.01	0.07 c	0.01	0.23 b	0.01	0.07 c	<0.01	0.23 b	0.02
C15	0.51 a	0.01	0.56 a	0.01	0.51 a	0.03	0.31 b	0.01	0.36 b	0.05	0.14 c	0.02
C16	52.54 a	0.03	48.93 b	0.02	35.88 c	0.08	30.33 C	0.03	25.49 d	0.04	31.33 c	0.01
C16:1 c7 n9	0.06 d	<0.01	0.13 c	0.02	0.06 d	0.02	0.20 b	0.00	0.12 c	0.01	0.70 a	0.03
C16 Phy	1.23 c	0.06	0.33 d	0.01	1.29 c	0.03	1.18 c	0.13	3.15 a	0.08	1.62 c	0.06
C16:1 c9	0.29 e	0.07	1.25 c	0.01	0.96 d	0.02	6.08 a	0.13	2.17 b	0.09	1.25 c	0.08
C16:2 c9 t12	<LOD e	—	<LOD e	—	0.04 d	<0.01	0.09 c	0.01	0.22 b	0.01	1.06 a	0.14
C17	0.27 a	0.01	0.12 B c	0.01	0.11 c	<0.01	0.09 c	0.02	0.14 b	0.04	0.10 c	0.01
C16:2 c9 c12	<LOD e	—	0.39 b	<0.01	0.08 c	<0.01	0.67 a	0.02	0.13 c	0.03	<LOD d	—
C17:1 c10	0.13 b	0.01	0.14 b	0.01	0.10 b	<0.01	0.15 ab	0.01	0.17 a	<0.01	0.16 ab	0.02
C16:3 c7 c10 c13 n3	<LOD d	—	<LOD d	—	<LOD d	—	0.22 b	0.02	0.03 c	<0.01	10.77 a	0.01
C18	1.56 a	0.03	0.94 b	0.04	1.10 b	0.02	0.47 c	0.01	0.78 d	0.01	0.62 d	0.01
C18:1 c9 n9	11.13 b	0.39	12.51 a	0.00	6.54 d	0.01	8.69 c	0.02	11.88 b	0.78	12.57 a	0.01
C18:1 c11	1.41 c	0.02	3.90 a	0.02	2.12 b	0.17	0.46 c	0.02	2.32 b	0.07	1.01 d	0.01
C18:2 c9 c12 n6	0.85 d	0.05	1.43 b	0.01	1.79 c	0.05	5.73 a	0.01	5.08 b	0.48	5.12 b	0.01
C18:3 t9 t12 c15 n3	0.22 d	0.03	0.32 c	0.03	0.79 a	0.02	0.51 b	0.02	0.03 e	<0.01	0.06 e	<0.01
C18:3 c6 c9 c12 n6	0.34 d	0.01	0.31 d	0.02	0.07 e	<0.01	8.87 a	0.01	0.65 c	0.03	2.84 b	0.14
C18:3 c9 c12 c15 n3	0.11 b	0.02	<LOD d	—	0.10 b	<0.01	0.34 c	0.01	6.47 b	0.07	17.38 a	0.02
C20	0.11 d	0.01	0.16 c	0.01	0.07 e	<0.01	5.18 a	0.01	0.59 b	<0.01	0.16 c	0.01
C20:1 c9	0.22 d	0.02	0.08 d	<0.01	0.09 d	<0.01	2.09 b	0.01	11.09 a	0.23	1.97 b	0.01
C20:1 c11 n9	0.46 c	0.03	0.55 b	0.02	1.03 a	0.01	0.58 b	0.05	0.07 d	0.01	<LOD e	—
C20:3 c11 c14 c17 n3	0.35 c	0.05	<LOD d	—	0.47 a	0.13	<LOD d	—	0.53 a	0.01	0.51 a	0.03
C20:4 c5 c8 c11 c14 n6 AA	18.62 a	0.50	4.92 d	0.01	12.26 a	0.05	12.43 c	0.04	15.01 b	0.03	2.86 e	0.01
C22	<LOD b	—	<LOD b	—	<LOD b	—	<LOD b	—	<LOD b	—	1.73 a	0.01
C22:1 c9	<LOD e	—	<LOD e	—	0.23 c	0.02	2.30 a	0.04	0.54 b	0.01	0.25 c	0.00
C20:5 c5 c8 c11 c14 c17 n3	<LOD f	—	15.58 b	0.14	29.92 a	0.02	7.54 c	0.02	5.77 d	0.02	1.67 e	0.02
C22:2 c13 c16 n6	<LOD b	—	<LOD b	—	0.22 a	0.01	<LOD b	—	<LOD b	—	<LOD b	—
C24	0.17 b	0.01	<LOD e	—	0.05 d	0.01	0.20 b	0.02	0.11 c	0.01	0.93 a	0.02
C22:5 c4, c7, c10, c13, c16 n6	<LOD c	—	<LOD c	—	<LOD c	—	0.18 b	0.01	0.31 a	0.01	<LOD c	—
C24:1	0.44 a	<0.01	0.13 c	0.03	0.18 b	0.02	<LOD d	—	0.16 bc	0.04	<LOD d	—
C22:5 c7 c10 c13 c16 c19 n3	0.61 a	0.09	<LOD c	—	0.06 b	<0.01	<LOD c	—	<LOD c	—	<LOD c	—
SFA (%)	63.54 a	0.24	58.07 a	0.03	42.74 b	0.19	42.17 b	0.19	36.42 c	0.16	38.88 bc	0.02
MUFA (%)	15.24 d	0.34	18.92 c	0.13	11.54 e	0.03	21.13 b	0.15	29.09 a	0.08	18.51 c	0.08
PUFA (%)	21.22 c	0.58	23.01 c	0.03	45.72 a	0.16	36.70 b	0.01	34.49 b	0.07	42.60 a	0.10
Total n9 (%)	11.78 b	0.33	13.10 a	0.11	7.61 d	0.01	9.35 c	0.03	12.20 b	0.89	12.77 ab	1.36
Total n6 (%)	20.14 b	0.16	6.68 e	0.02	14.41 c	0.06	27.46 a	0.30	21.48 b	0.13	10.99 d	0.19
Total n3 (%)	1.38 e	0.44	16.08 b	0.13	31.56 a	0.11	8.88 d	0.17	13.21 c	0.03	31.57 a	0.72
n6/n3	15.36 a	1.83	0.42 de	0.01	0.46 d	0.05	3.09 b	0.44	1.63 c	0.14	0.35 e	0.02
µg FA/$mg_{dry\ seaweed}$	12.31 d	0.09	16.47 c	0.31	20.89 b	0.59	17.30 c	0.44	19.96 b	0.14	27.58 a	0.15

Data expressed as mean (Mean; n=3) and standard deviation (SD). ai: anteiso. Phy: Phytanic acid. AA: Arachidonic acid. c/t: cis/trans double bond. SFA/MUFA/PUFA: Percentage of total of saturated/monounsaturated/polyunsaturated fatty acids; n3, n6 and n9: Omega-3, -6 and -9 fatty acids. DM: dry matter. a-f: in a row, significant differences among seaweed species.

On the other hand, the amounts of oleic acid (C18:1 c9) in *O. pinnatifida*, *G. gracilis*, *C. tomentosum* and *S. polyschides* (11.33 to 12.57 /100g_{fat}) may compensate the contents in AA as it has been described to exert anti-inflammatory activities.

Other works comparing the composition of several seaweeds including that from *G. gracilis, G. turuturu* and *O. pinnatifida* reported a qualitative FA profile that is characteristic of these samples due to the presence of C18:3 c9 c12 c15 and AA (Schmid et al., 2014; Kendel et al., 2013; Patarra et al., 2013). Those studies also reported similar compositions to that of the current study.

It must not be forgotten that at the current moment the elevated intake of seed oils plays a central role in the unbalance of the n6/n3 ratio and the development of CVD and CHD. According to WHO (2008), the ratio of n6/n3 should be lower than 10 in the diet. Ratio values lower than 1 were observed in *O. pinnatifida*, *G. turuturu* and in *C. tomentosum* (Table 3.2) making these edible species, already consumed in several countries such as Malaysia, China, Japan or Ireland (Munier et al., 2013; Roo et al., 2007), particularly adequate to be incorporated in a more balanced diet, from a lipid point of view. A ratio of 0.31 was reported by Paiva et al. (2014) for *O. pinnatifida*. A particularly high value for n6/n3 ratio was observed in *G. gracilis* in part due to the absence of EPA.

3.3.3. Elemental composition of seaweeds

Seaweeds are known to be rich in macroelements with some content in trace elements. The elemental composition of the six seaweed species was analysed and is displayed in Figure 3.1a-f. The most significant macroelements present in the seaweeds were K, Na, Mg and Ca, accounting for more than 97 % of the total mineral content, demonstrating that seaweeds are a good source of these elements. Phosphorous was present at a fairly constant value (between 1.72 and 2.81 mg/$g_{dry\ seaweed}$) among all 6 species of seaweeds studied but of much lower order of magnitude than the four other minerals previously mentioned.

In what concerns mineral distribution per species, inter and intra seaweed class variations were observed. For example, K was the most predominant element found in *S. muticum*, *G. gracilis* and *S. polyschides* with contents ranging between 16.28 to 76.54 mg/$g_{dry\ seaweed}$, being statistically different (p<0.05) among all the six species under study. Sodium content also differed significantly between the different seaweed species except between *O. pinnatifida* and *G. turuturu* (p>0.05). In fact, in *O. pinnatifida* and *G. turuturu* Na was the most predominant element ranging between 92.88 and 96.08 mg/$g_{dry\ seaweed}$ yet it attained an extremely high value of 204.52 mg/$g_{dry\ seaweed}$ in *C. tomentosum* (also most predominant element). Sodium/potassium ratios lower than 1 were observed for *G. gracilis* (0.3), *S. muticum* (0.6) and *S. polyschides* (0.9) whereas ratios higher than 1 were observed for *O. pinnatifida* (3.56), *G. turuturu* (5.9) and C*. tomentosum* (5.5), respectively.

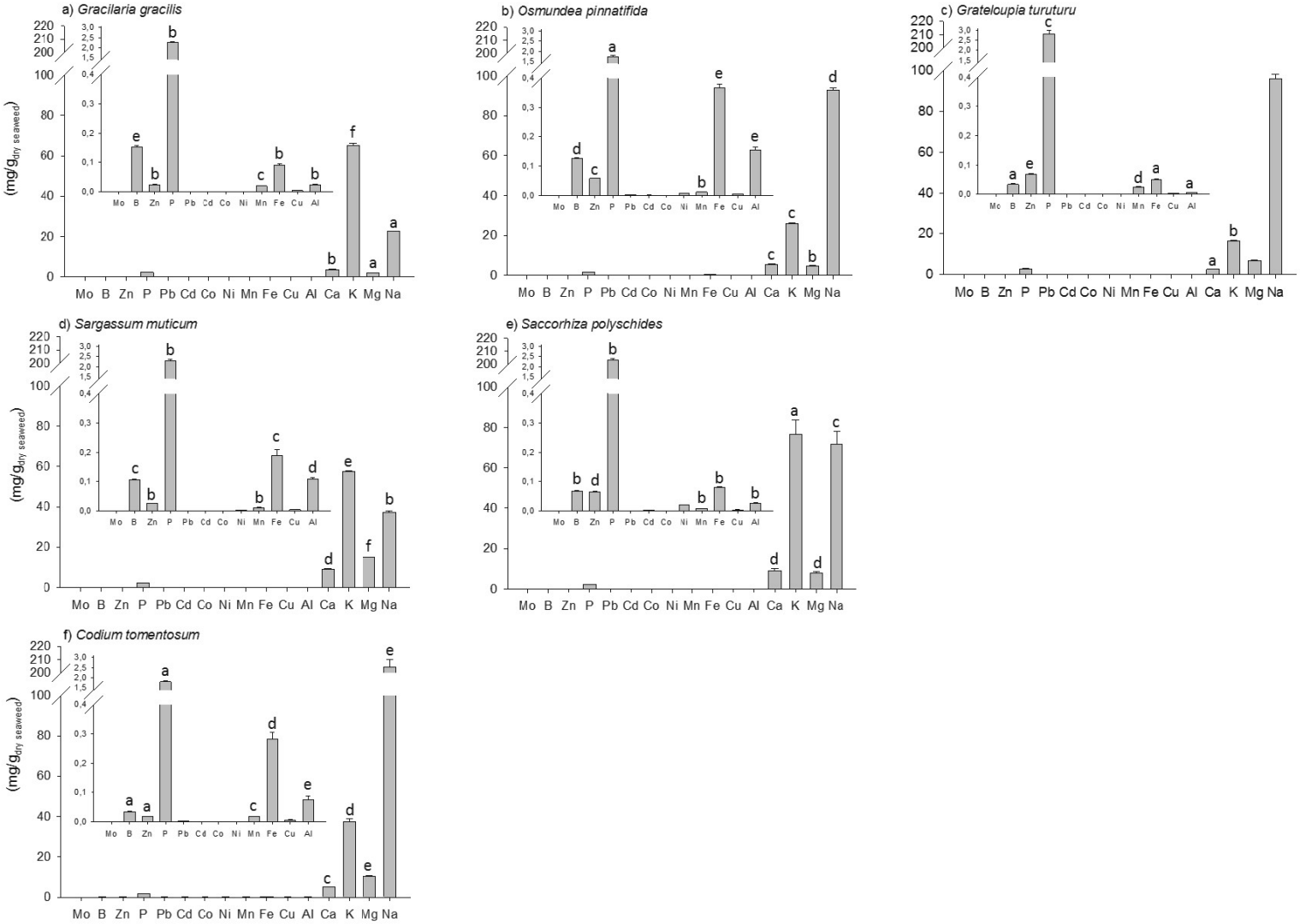

Figure 3.1. Element contents (mg/$g_{dry\ seaweed}$) in the different species of red (Rhodophyta) (a, b and c), brown (Phaeophyceae) (d and e) and green (Chlorophyta) (f) seaweeds. For each element, Different letters indicate significant differences (p<0.05) between species.

A similar tendency was reported by Jard et al. (2013) for *S. muticum* (0.6), *S. polyschides* (0.4) *and C. tomentosum* (10.0). Lower ratio Na/K was reported by Paiva et al. (2014) for *O. pinnatifida* (1.82). Seaweeds with low ratios of Na/K are good possibilities to be used as salt substitutes.

Magnesium is known to be an important mineral for cardiovascular function (Krishnaiah et al., 2008) and its external administration could prevent its intracellular depletion. Magnesium content in the six seaweeds ranged between 1.75 $mg/g_{dry\ seaweed}$ for *G. gracilis* and 15.04 $mg/g_{dry\ seaweed}$ for *S. muticum* (Figure 3.1a-f) differing significantly between each species (p<0.05); in general, contents were higher in species of brown and green seaweed classes than in red seaweed species.

Calcium values ranged between 2.65 $mg/g_{dry\ seaweed}$ in *G. turuturu* to 9.11-9.18 $mg/g_{dry\ seaweed}$ in brown seaweeds *S. muticum* and in *S. polyschides*, respectively. Calcium content was not statistically different (p<0.05) among *S. muticum* and *S. polyschides* as well as among *C. tomentosum* and *O. pinnatifida*. Calcium is well-known for its importance in terms of health preservation due to its diverse biological roles being essential for structural support, cell adhesiveness, mitosis, blood coagulation, muscle contraction and glandular secretion being the most significant consequence of the low calcium status the occurrence of osteoporosis, which is a disease characterized by reduced bone mass which increases skeletal fragility (Allen et al., 2006).

Phosphorous was the least representative macroelement in quantitative terms, ranging between 1.72-2.80 $mg/g_{dry\ seaweed}$. No significant differences for P were observed between *S. polyschides*, *S. muticum* and *G. gracilis* (p>0.05) as well as between *C. tomentosum* and *O. pinnatifida* (p>0.05). 1 to 2 $g/Kg_{dry\ seaweed}$ of P was reported by Jard et al. (2013) for *S. muticum*, *S. polyschides* and *C. tomentosum*. Phosphorus is part of the skeletal structure and teeth having other important functions such as the contribution to the control of acid-base balance in the blood; in the metabolism of carbohydrates it contributes to the intestinal absorption of glucose by the process of phosphorylation (Pérez et al., 2005).

In terms of microelements B, Zn, Mn, Fe and Al were the most representative among the six species of seaweeds analysed. In the six seaweeds species Fe was the most representative among the microelements especially in *S. muticum*, *O. pinnatifida* and *C. tomentosum* ranging from 0.05 and 0.37 $mg/g_{dry\ seaweed}$; values were statistically different among all species analysed except for comparable contents among *G. gracilis* and *S. polyschides*. Iron is a component of various enzymatic systems and has as its main function the transport of oxygen from the lungs to the tissues: Iron deficiency is one of the most common nutritional disorders worldwide due to several reasons such as bleeding or mal-nutrition balance. It is known that iron deficiency can cause anaemia (Allen et al., 2006) and therefore infants under 24 months of age when exposed to iron deficiency can be affected in the development of the neuropsychomotor system, with severe consequences for the future. Zinc element which ranged from 0.02 $mg/g_{dry\ seaweed}$ in *C. tomentosum* to 0.07 $mg/g_{dry\ seaweed}$ in *G. turuturu* is known to enhance the catalytic, structural and regulatory functions, to stabilize membranes, hormones and nucleic acids (Krishnaiah et al., 2008), and is important for cellular growth and differentiation in tissues that have a rapid differentiation and turnover (Allen et al., 2006). The deficiency of Zn could be a limiting factor during pregnancy

affecting normal embryonic and fetal growth in experimental animals, and also the length of gestation.

In general, the six edible seaweeds collected from the Portuguese coast were of important nutritional value not only from its proximate composition but especially due to its elemental composition. As far as the Portuguese population is concerned these analysed seaweeds available along the Portuguese coast offer a wide array of nutrients, at concentrations that may meet with nutritional requirements, particularly in what concerns elements present and in some cases may even allow for application of nutritional claims. Data on daily intake of seaweeds are unavailable for the Portuguese population let alone for other European countries. Nonetheless, if seaweed daily consumption in Asian countries is taken into account then one may consider 8-10 g of seaweed dry matter as an average daily intake (Mišurcová et al., 2011). Based on this reference value Table 3.1 indicates the possible contribution of analysed seaweeds to daily nutritional requirements. Whereas such daily intake contributes with a small fraction to protein, carbohydrate and fat requirements (Table 1), in terms of microelements some of these seaweeds contribute well over 15% (minimum requirement for nutritional claim) of the recommended daily intakes (RDIs). Variable contribution to the RDI for the elements is observable in Table 3.1. In terms of macroelements all species except *G. turuturu* are good contributors to potassium RDI, especially *G. gracilis* and *S. polyschides* with values higher than 30%. All selected species, except *G. gracilis*, can be considered as good sources of Mg, especially *S. muticum* (40.1%). On the other hand these species were low providers of Ca and P. Interesting contribution values to Fe RDI were observed in *O. pinnatifida*, *S. muticum* and *G. turuturu* with values ranging between 13 to 26%. Lower values were, in general, found for the other microelements. Higher values of %RDI for Fe, Zn and Mn were, in general, reported by Mišurcová et al. (2011) but for other brown, red and green seaweed. According to these authors seaweeds could be utilized as nutraceuticals due to their richness in elements. As previously mentioned, an adequate intake of minerals is essential to prevent nutritional deficiencies and diseases but excessive intakes of trace elements may cause toxicity.

3.3.4. FTIR-ATR and FT-Raman characterization

Vibrational spectroscopy can reveal detailed information concerning the properties and structure at a molecular level for compounds such as carrageenans and agar present in some red seaweed (Rhodophyceae), alginic acid/alginates present in some brown seaweeds (Phaeophyceae) (Pereira, 2006). In seaweeds sugars, followed by protein (Table 3.1), are the major groups of compounds. Carrageenans and agar (galactans) are the main sulphated polysaccharides produced by red seaweeds (Rhodophyta), whereas alginate is mainly found in brown seaweeds (Phaeophyceae). Fucans including compounds such as fucoidin, fucoidan, sargassan, etc. are sulphated polysaccharides that can also be found in brown seaweeds (Shanmugan & Mody, 2000). The major polysaccharides in green seaweeds (Chlorophyta) are, in turn, polydisperse heteropolysaccharides

where glucuronoxylorhamnans, glucuronoxylorhamnogalactans or xyloarabinogalactans are the three main groups (Shanmugan & Mody, 2000).

According to Gómez-Ordóñez & Rupérez (2011), based on Mathlouthi & Koenig (1987) verifications, five frequency regions can be differentiated in the normal spectra (4000-650 cm^{-1}) from vibrational structural analysis of carbohydrates for seaweeds: (1) region of O-H and C-H stretching vibrations at 3600-2800 cm^{-1}; (2) region of local symmetry at 1500-1200 cm^{-1}; (3) region of C-O stretching vibration at 1200-950 cm^{-1}; (4) fingerprint or anomeric region at 950-750 cm^{-1}; and (5) skeletal region below 700 cm^{-1}. N-H stretching vibrations at 3700-2900 cm^{-1} as well as from amide I and amide II at 1700-1420 cm^{-1} could be related to proteins (Chopin et al., 1999) which were found in a range between 14.3 and 23.8 $g/100g_{dry\ seaweeds}$ in the selected seaweeds.

In Figure 3.2, it is possible to observe two bands in the 4000-2000 cm^{-1} common in all studied seaweeds, which is in accordance to data published by Gomez-Ordóñez & Rupérez (2011) for phycocolloid standards (carrageenans, agar and alginate) as well as for seaweeds samples. A broad band at 3280-3350 cm^{-1} and a weaker signal at 2870-2960 cm^{-1} could be assigned to O-H and C-H stretching vibrations but also to N-H stretching vibrations, respectively.

Medium to strong IR absorption bands were observed in all spectra between 900 and 1750 cm^{-1} in FTIR-ATR spectra. According to several authors, bands between 1200 and 970 cm^{-1} are due mainly to C-C and C-O stretching bonds, common to all polysaccharides (Gomez-Ordóñez & Rupérez 2011). Pereira et al. (2013) focused their analysis by FTIR-ATR and by FT-Raman of seaweed polysaccharides mainly in the 600-1500 cm^{-1} spectra range. In figures 3.3a to 3.3c, the 700-1400 cm^{-1} regions of all seaweeds are amplified enabling a more detailed observation of the characteristic bands present in this region. FTIR, FTIR-ATR and FT-Raman band assignments by several authors for red (Rhodophyta) seaweeds polysaccharides (carragenans and agar) and for brown (Phaeophyta) seaweeds polysaccharides (alginates and fucoidans) are listed in Table 3.3, respectively.

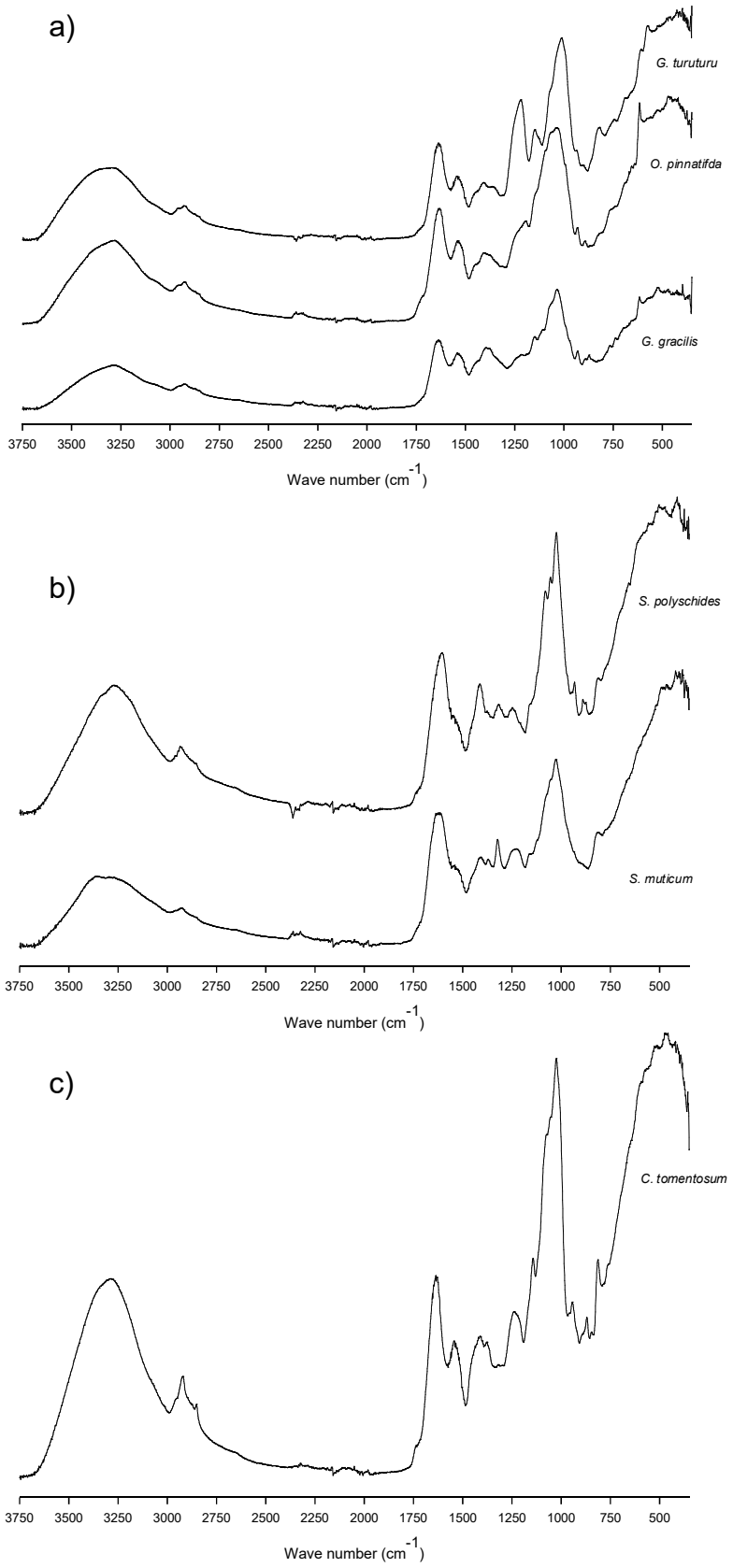

Figure 3.2. FTIR-ATR spectra of red (Rhodophyta) (a), brown (Phaeophyceae) (b) and green (Chlorophyta) (c) seaweeds.

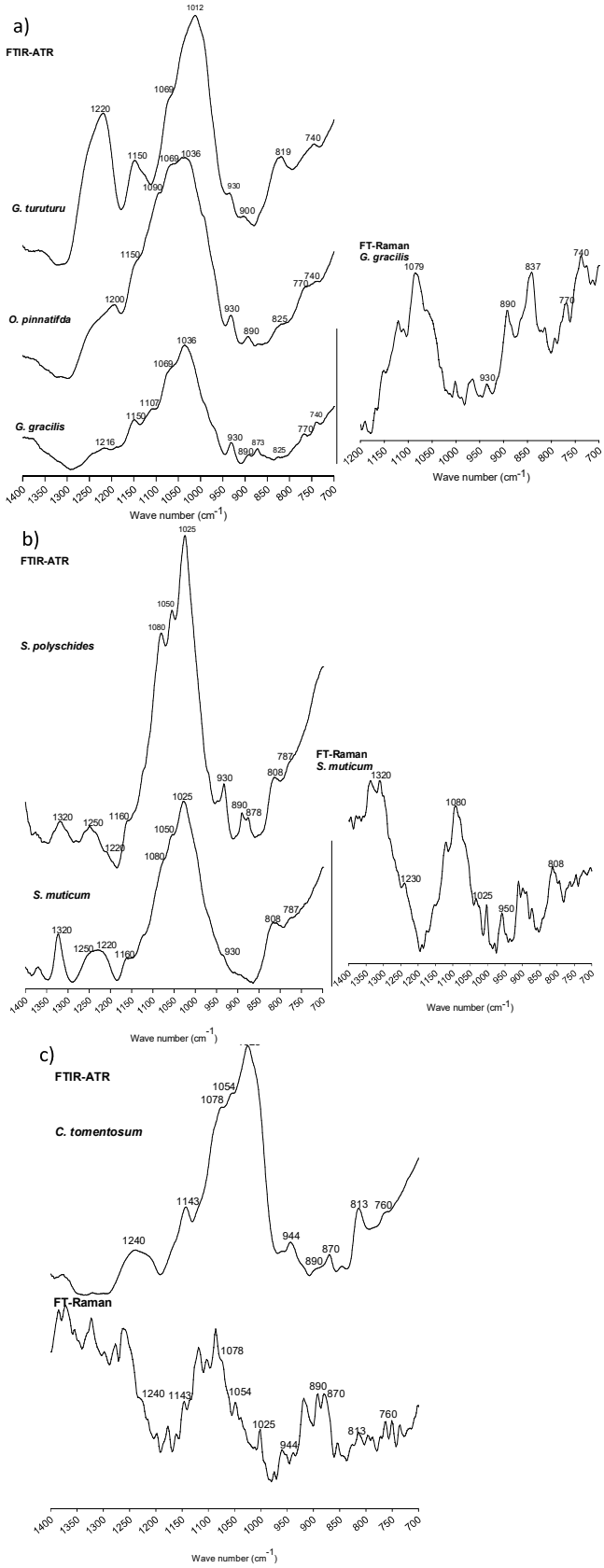

Figure 3.3. FTIR-ATR and FT-Raman spectra of red (Rhodophyta) (a), brown (Phaeophyceae) (b) and green (Chlorophyta) (c) seaweeds between 1400 and 700 cm-1.

Table 3.3. FTIR-ATR and FT-Raman band assignment for polysaccharides from red (Rhodophyta) and brown (Phaeophyceae) seaweeds.

	Wave numbers (cm^{-1})	Assignment*	Vibrational Spectroscopy	Polysaccharides	References
Red seaweeds	1210-1260	S=O of sulphate esters	FTIR, FTIR-ATR, FT-Raman	Carrageenan Agar	Knutsen et al., 1994; Pereira, 2006; Gómez-Ordónez & Rupérez, 2011
	1150	Sulphate ester	FTIR-ATR	Carrageenan Agar	Chopin et al. ,1999
	1070-1085, 925-935; 837	C-O of 3,6-anhydro-D-galactose	FTIR, FTIR-AT, FT-Raman	Carrageenan Agar	Knutsen et al., 1994; Pereira et al., 2003, 2013; Pereira, 2006; Gómez-Ordónez & Rupérez, 2011
	1010-1030	C-O, C-C stretching vibration of pyranose ring	FTIR, FTIR-AT, FT-Raman	Carrageenan Agar	Pereira et al., 2013
	970-975	D-galactose	FTIR; FT-Raman	Carrageenan	Knutsen et al., 1994; Pereira, 2006
	905-907, 800-808	C-O-SO3 on C2 of 3,6-anhydro-galactose	FTIR; FTIR-ATR, FT-Raman	Carrageenan	Knutsen et al., 1994; Pereira, 2006; Gómez-Ordónez & Rupérez, 2011
	890-900	C-H on anomeric carbon of β-galactose (unsulphated β-D-galactose)	FTIR-ATR FT-Raman	Carrageenan Agar	Pereira et al., 2003, 2009; 2013
	867-871, 810-825	C-O-SO_3 on C_6 of galactose	FTIR; FTIR-ATR, FT-Raman	Carrageenan	Knutsen et al., 1994; Pereira, 2006; Gómez-Ordónez & Rupérez, 2011
	840-850	C-O-SO_3 on C_4 of galactose	FTIR; FT-ATR; FT-Raman	Carrageenan	Knutsen et al., 1994; Pereira et al., 2003, Pereira, 2006; Gómez-Ordónez & Rupérez, 2011
	820-830	C-O-SO_3 on C_2 of galactose	FTIR; FTIR-ATR, FT-Raman	Carrageenan	Knutsen et al., 1994; Pereira, 2006; Gómez-Ordónez & Rupérez, 2011
	740, 770	Skeletal bending of galactose ring	FTIR-ATR FT-Raman	Carrageenan Agar	Pereira et al., 2003; 2013
Brown seaweeds	1195-1260	S=O of sulphate esters	FTIR-ATR	Fucoidan	Gómez-Ordónez & Rupérez, 2011; Pereira et al., 2013
	1290, 1080, 1025, 787	Guluronic acid	FTIR-ATR; FT-Raman	Alginate	Gómez-Ordónez & Rupérez, 2011; Pereira et al., 2013;
	1320,1030, 1019, 808	Mannuronic acid	FTIR-ATR; FT-Raman	Alginate	Gómez-Ordónez & Rupérez, 2011; Pereira et al., 2013;
	948	C-O of uronic acid residues	FTIR-ATR	Alginate	Gómez-Ordónez & Rupérez, 2011
	878	C_1-H of β-mannuronic acid	FTIR-ATR	Alginate	Gómez-Ordónez & Rupérez, 2011

*Band assignments resulted both from the use of standards (agar and carrageenans for the red seaweeds and alginate for the brown seaweeds) as well as from the red or brown seaweeds studied by the referenced authors.

The FTIR-ATR spectrum of *G. turuturu* in the region 1400-700 cm^{-1} (Figure 3.3a) show some characteristic features related with typical polysaccharides from red seaweeds (Table 3.3):
i) a high absorbance band at 1220 and 1150 cm^{-1}, typically more intense for highly sulphated polysaccharides (such as carrageenans) than in less sulphated polysaccharides (such as agar), and at 1012 cm^{-1} which have been assigned to S=O sulphate esters and to C-O and C-C stretching vibrations of pyranose common to all polysaccharides, respectively; Strong absorption at 1220-1260 cm^{-1} have been reported by Yu et al. (2012) for agaran-type polysaccharide isolated from *Grateloupia filicina*;
ii) 930 and 900 bands in the anomeric region (950-700 cm^{-1}) assigned to the presence of 3,6-anhydro-D-galactose and anomeric C-H of β-galactopyranosyl residues, respectively; the first band has been found in carragenan and agar polysaccharides (Pereira, 2006) whereas the second is more typical in Beta carrageenans (Pereira, 2006; Knutsen et al., 1994);
iii) a relatively strong band at 819 cm^{-1} which has been assigned to the presence of galactose units sulphated at the C-2 position, typical in lambda carrageenans standards spectra (Pereira et al., 2009) but also observed in agaran-type polysaccharide isolated from *G. filicina* (Yu et al. 2012).

According to several reported studies, natural diversity of polysaccharides among *Grateloupia* spp. exists; polysaccharides isolated from other *Grateloupia* species have showed agaran-carrageenan backbones (*G. indica*, Sen et al., 2002) or agaran-type backbone (*G. filicina*, Yu et al., 2012). Enzymatic digestion of *G. turuturu* confirmed indirectly the presence of agar, kappa and iota carrageenans in their cell walls (Denis et al., 2009). Therefore, and in accordance to the main bands observed in *G. turuturu* spectrum (Figure 3.3a), this red seaweed may be a producer of agaroid-carrageenan hybrid polysaccharides.

The phycocolloid agar is obtained from some families of red seaweeds such as Gracilariaceae and Gelidiaceae; *Gracilaria* species, namely *G. gracilis*, are known to produce agars with relatively high sulphate content. According to Pereira et al. (2013), agars differ from carrageenans as they have a L- configuration for the 4-linked galactose residue but they have some structural similarities with carrageenans. In fact the FTIR-ATR spectrum obtained for *G. gracilis* presented several bands in common to the *G. turuturu* spectrum. Some of the main differences were centred on the broad band at 1220 cm^{-1} and at 1020-1040 cm^{-1} which were extremely more intense in the *G. turuturu* spectrum. The band at 890 cm^{-1} has been assigned to unsulphated β-D-galactose, whereas, band at 825 cm^{-1} has been assigned to α-L-galactose sulphate residues, typical of agar type seaweeds. The strong bands at 740 and 770 cm^{-1} in FT-Raman spectrum and weak in FTIR-ATR spectrum (Figure 3) are according to those observed by Pereira et al. (2003) for red agar-like seaweed. 740 and 770 cm^{-1} bands are assigned to the skeletal bending of the galactose ring (Table 3) whereas 890 cm^{-1} is typical of agar being associated with C-H bending at the anomeric carbon of β-galactose residue (Table 3.3). The strong bands at 837 and 1079 cm^{-1} in FT-Raman spectrum, absent in FTIR-ATR, have been observed by Pereira et al. (2003) in commercial agar and in *G. gracilis* extracted agar.

Due to the high similarity of the *O. pinnatifida* spectrum with that of *G. gracilis*, especially in terms of such diagnostic agar bands as those at 1220, 1020-1040 and 890 cm^{-1} in FTIR-ATR spectrum and 1079, 890, 837, 770 and 740 cm^{-1} in FT-Raman spectrum (data not shown), it could be indicated that this species probably may be considered an agar-like producer.

The main polysaccharide which have been found in brown seaweeds such as *S. muticum* and *S. polyschides* is alginate known to be a linear copolymer of β-D-mannuronic acid and α-L-guluronic acid (1-4)-linked residues arranged in heteropolymeric and/or homopolymeric blocks (Pereira et al. 2003). The presence of these acids can be evidenced especially by the bands at 1025, 1080 and 787 cm^{-1} assigned to guluronic acid and at 808 and 1320 cm^{-1} assigned to mannuronic acid, respectively (Figure 3.3b, Table 3.3). A strong band at 1025 cm^{-1} in the FTIR-ATR spectrum and weak in FT-Raman spectrum was observed by Pereira et al. (2013) in a commercial alginate and in *S. polyschides*. In addition, this band is indicative that both *S. muticum* and *S. polyschides* are particularly rich in guluronic acid. The FTIR-ATR spectrum of *S. muticum* suggests that this species is richer in mannuronic acid in comparison to *S. polyschides* due to more intense bands at 808 and 1320 cm^{-1}. According to Pereira et al. (2013), *Sargassum vulgare* was characterized by similar amounts of both mannuronate and guluronate residues. The broad band around 1220-1260 cm^{-1} in FTIR-ATR spectrum, assigned to the presence of sulphate ester groups (S=O) which is a characteristic component in fucoidan and other sulphated polysaccharides that can be found in some brown seaweeds, are observable for both *S. polyschides* and S. *muticum* (Figure 3.3b). Fucans comprise heteromolecules based on L-fucose, D-xylose, D-glucuronic acid, D-mannose and D-galactose (Shanmugan & Mody, 2000).

(1→4)-β-D-mannans have been identified as the predominant skeletal wall polysaccharides in green seaweeds including in the *Codium* genera (Dunn et al., 2007). In terms of matrix cell wall polysaccharides, sulphated xyloarabinogalactans but also a variable range of these polymers such as sulphated galactans, sulphated arabinan, sulphated arabinogalactans and sulphated glucans have been found in green seaweeds (Estevez et al., 2009). Sulphated arabinan and sulphated arabinogalactan have been identified in *C. tomentosum* (Shanmugan & Mody, 2000). In figure 3.3c more intense bands are observable at 1143, 1078, 1054 and 1025 cm^{-1} which could probably be assigned to (1→4)-β-D-mannans. Estevez et al. (2009) reported FTIR spectrum bands at 1151, 1092, 1061 and 1032 cm-^{1} associated to β-mannans in *Codium fragile*; 1151 cm^{-1} band has been assigned to the glycosidic C-O-C vibration of (1→4)-β-D-mannans whereas the bands at 1061 and 1032 cm^{-1} have been assigned to C-O-C and C-C bonds of mannose unit rings. Bands at 1200-1000 cm^{-1} region have been assigned to sugar rings vibration overlapping with stretching of side groups (C-OH) and glycosidic bonds vibration (C-O-C) in Ulvophyceae green seaweeds (Pereira & Ribeiro-Claro, 2014). A broad weak band around 1240 cm^{-1} is typical of S=O of sulphate esters of sulphated polysaccharides which is observable in the spectrum of C. *tomentosum* (Figure 3.3c), indicating the presence of this type of polysaccharide in this green seaweeds. Bands between 945 and 760 cm^{-1} with different intensities in FTIR or FT-Raman spectra (Figure 3.3c) are probably due

to the presence of sulphated and unsulphated galactose residues. Scarce information on vibrational spectroscopy analysis on *Codium* specimens is found in the literature.

3.4. Conclusions

The study shows that the six edible seaweeds species harvested from the Portuguese West coast have a good potential for further processing or for direct food and nutraceutical applications given their very good nutrient profiles. The proximate and elemental composition varied significantly among brown, red and green seaweeds as well as within species in each main class. Red seaweeds species registered the highest protein content but the lowest fat and sugar content. On the other hand, comparatively green and brown seaweeds stood out for their highest fat and sugar content, respectively. The low fat content (0.6 to 3.6%) found among the studied seaweeds was coupled to a specific FA profile rich in palmitic acid, araquidonic acid and EPA; the presence of phytanic acid was also worth noting. Higher total phenolic content was observed in the green seaweeds (*C. tomentosum*) followed by brown seaweeds (*S. muticum*) and the red seaweeds (*O. pinnatifida*). In general, seaweeds were characterized by high levels of minerals. Higher levels of ash were associated with higher amounts of elements content especially of macroelements. Some of the selected seaweeds (*S. muticum*, *S. polyschides* and *C. tomentosum*) may be included in human diet to help solve problems with mineral deficiency, in particular, Ca, K, Mg and Fe, since they revealed to be good sources of these elements contributing significantly to the daily requirements intake of several countries. According to FTIR-ATR and FT-Raman spectra, *G. gracilis* and *O. pinnatifida* were mostly agar producers whereas *G. turuturu* was associated *to* agaroid-carrageenan hybrid polysaccharides. In the brown seaweeds, *S. muticum* and *S. polyschides,* alginates and fucoidans were the main representative polysaccharides and in *C. tomentosum* (1→4)-β-D-mannans, sulphated and unsulphated galactose residues were evidenced. The presence of these polysaccharides further upholds the interest in exploring these seaweeds for applications in health-related fields, for example, drug or nutraceutical delivery approaches.

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Chapter 4

Chemical composition and nutritive value of a selection of cultivated and edible mushrooms

4.1. Introduction

Edible mushrooms have been appreciated ever since early times for their sensory characteristics and culinary suitability. Furthermore, they are well recognized for their nutritional and health benefits as described in chapter 2. There are several hundreds of wild species of edible mushrooms yet only about 20 species are cultivated and used more extensively as food (Kalač, 2013) and only 10 species are cultivated on an industrial scale (Reis et al., 2012a). The most commonly eaten species are *Agaricus bisporus* (Paris mushroom) and *Lentinula edodes* (Shiitake mushroom). The 'Shiitake mushroom' is as common in Asian countries as *Agaricus bisporus* is in the western world (Ghorai, 2009). Although cultivated mushrooms species has grown recently, there is still a lack of knowledge on their chemical composition which is essential to assess their nutritional value as well as their potential for functional ingredients provision. Therefore, the main objective of the study presented in this chapter was to determine the chemical composition of five cultivated edible mushrooms, some less characterized such as *Pleurotus citrinopileatus* var. *cornucopiae* or *Pleurotus salmoneo stramineus, Pholiota nameko* and *Hericium erinaceus*. Information on the proximal composition, fatty acids (FA) profile, elemental composition and main chemical compounds identified by FTIR-ATR of the five cultivated mushrooms is presented and discussed as well as their nutritive value and possible role in a healthy diet. According to my knowledge, there are no previous studies describing the FA composition of the edible mushrooms *P. salmoneo stramineus, P. citrinopileatus* var *cornucopiae*, *Ph. nameko* and *H. erinaceus*.

4.2. Material and methods

4.2.1. Mushrooms species and cultivation conditions

Dried specimens of cultivated mushrooms were supplied by Bioinvitro, Biotecnologia, Lda. **(**Gandra, Portugal): three specimens of order Agaricales and family Pleurotaceae, *Pleurotus citrinopileatus* var. *cornucopiae, Pleurotus eryngii* and *Pleurotus salmoneo stramineus*; one specimen of order Agaricales and family Strophariaceae, *Pholiota nameko*; and one specimen of order Russulales and family Hericiaceae, *Hericium erinaceus.* The classification of mushrooms was based on MycoKey™ (Petersen & Læssøe, 2013). Mushrooms were cultivated in filter bags with sterilized organic substrate. Table 4.1 summarizes the cultivation conditions (Bioinvitro information) for each species of mushroom. After fructification and growth, entire clean mushrooms were dried in a ventilated drier through 24h between 40-60 ºC. The dried mushrooms were subsequently milled to less than 1.0 mm particle size.

Table 4.1. Summary of the conditions for **each of the cultivated mushroom species.**

Mushroom	Organic substrate[1]	Water content[2]	Incubation	Fructification
Pleurotus citrinopileatus var. cornucopiae	Sawdust spruce, 38% Wood shavings, 5% Fibers (wood, straw), 20% Crushed grain corn, 18% Wheat bran, 13% Crushed oil seed cake, 6%	68%	20-30 days 22 ºC	5-10 days 18-20 ºC
Pleurotus salmoneo stramineus				5-10days 20-22 ºC
Pleurotus eryngii	Sawdust spruce, 20% Wood shavings, 5% Fibers (wood, straw), 30% Sugar beet pulp,5% Crushed grain corn, 14% Wheat bran, 14% Crushed oil seed cake, 12%	67%	20-30 days 20-22 ºC	13-18 days 15-22 ºC
Hericium erinaceus	Sawdust beech, 70% Fibers (wood, straw), 5% Crushed grain corn, 10% Wheat bran, 12% Crushed oil seed cake, 3%	64%	30-40 days 20-22 ºC	6-10 days 16-20 ºC
Pholiota nameko	Sawdust beech, 76% Fibers (wood, straw), 5% Crushed grain corn, 9% Wheat bran, 7% Crushed oil seed cake, 3%	65%	30-40 days 20-22 ºC	15-21 days 16-20 ºC

[1]Composition based on percentage of dry matter of each organic component; [2]Water content based on fresh weight.

4.2.2. Proximate composition

The content in moisture, organic matter, ash, nitrogen, protein (using 4.38 as converting factor to protein; Kulshreshtha et al., 2013), total fat, total sugar as well as total polyphenols were determined according to procedures described in chapter 3, section 3.2.2.1.

4.2.3. Analysis of fatty acids

The analysis of the total fatty acid (FA) composition as well as identification and quantification of fatty acids were determined according to procedures described in chapter 3, section 3.2.2.2.

4.2.4. Elemental composition

The analysis of elemental composition was performed in two steps: microwave-assisted digestion followed by determination of the 15 elements using an inductively coupled plasma (ICP) optical emission spectrometer (OES) with radial plasma configuration according to procedures described in chapter 3, section 3.2.2.3. The accuracy of the method (microwave acid digestion and ICP-OES analysis) was assessed by analysis of certified reference material IPE 120 (*Agaricus bisporus*; WEPAL, Holland). Five replicates of reference material were subject to microwave digestion and analysed three times by ICP-OES. Recovery rates ranged between 84-105%.

4.2.5. FTIR-ATR Analysis

Samples of milled, dried mushroom material were analysed by Fourier Transform Infrared Spectroscopy with attenuated total reflectance (FTIR-ATR) according to procedures described in chapter 3, section 3.2.2.4.

4.2.6. Statistical analysis

One-way ANOVA was carried out with SigmaStat™ (Systat Software, Chicago, IL, USA), to assess differences between mushroom species in terms of proximate or elemental composition at a significance level of p=0.05. Normality and homogeneity were examined and the Holm-Sidak method was used for pairwise comparisons (p<0.05). FA data in turn, were analysed using the IBM SPSS Statistics v22 for Mac. Normality and homogeneity were examined and one-way ANOVA with the Bonferroni test for post-hoc analyses was applied to evaluate statistical differences between mushrooms (p<0.05).

4.3. Results and Discussion

4.3.1. Proximate composition of cultivated mushrooms species

Mushrooms are known for being rich in protein and polysaccharides and poor in fat content, which from a nutritional point of view is of particular interest especially for low calorie diets with low fat content. The composition reported for the five species under evaluation (Table 4.2) was no different from this trend, yet among them, between the different families and within the same family, significantly different compositions were found, in particular, in what concerns protein and organic matter contents. Differences in total fat content were less relevant and only *P. salmoneo stramineus* presented a significantly different value (p<0.05); in fact the lowest fat content among the five species studied. Within the same family, i.e. Pleurotaceae family, *P. eryngii* was characterized by the lowest total protein content (16.2 g/100$g_{dry\ mushroom}$) followed by the highest content in total sugar (64.9 g/100$g_{dry\ mushroom}$) and in total fat (3.4 g/100$g_{dry\ mushroom}$) whereas *P. salmoneo stramineus,* presented the highest protein content (26.6 g/100$g_{dry\ mushroom}$) but the lowest content in total sugar (52.7 g/100$g_{dry\ mushroom}$) and in total fat (2.3 g/100$g_{dry\ mushroom}$).

Table 4.2. Proximate composition of mushrooms and their percentage contribution in nutrients intake.

Parameter	*Pleurotus citrinopileatus var. cornucopiae*	*Pleurotus salmoneo stramineus*	*Pleurotus eryngii*	*Hericium erinaceus*	*Pholiota nameko*
% Moisture ($g/100g_{dry\ mushrooms}$)	10.69±0.05 c	11.10±0.01 d	9.47±0.08 a	10.11±0.5 b	11.91±0.01 e
% Total Protein ($g/100g_{dry\ mushrooms}$)	23.8±0.1 d	26.6±0.2 e	16.2±0.3 a	18.8±0.1 c	16.92±0.03 b
% Total sugars[1] ($g/100g_{dry\ mushrooms}$)	54.7	52.7	64.9	61.3	59.6
% Total Fat ($g/100g_{dry\ mushrooms}$)	3.5±0.1 b	2.3±0.1 a	3.4±0.1 b	2.9±0.1 b	3.2±0.1 b
Total phenolic content (μg cathecol equiv/$g_{dry\ mushrooms}$)	1140±90 c	464±12 a	709±36 b	753±4 b	675±44 b
% Organic matter ($g/100g_{dry\ mushrooms}$)	82.03±0.07 c	81.63±0.02 b	84.54±0.01 e	82.98±0.06 d	79.68±0.06 a
% Ash ($g/100g_{dry\ mushrooms}$)	7.29±0.12 c	7.27±0.01	5.99±0.08 a	6.91±0.01 b	8.40±0.06 d

		Pleurotus citrinopileatus var. cornucopiae		*Pleurotus salmoneo stramineus*		*Pleurotus eryngii*		*Hericium erinaceus*		*Pholiota nameko*	
Nutrients	g/day	$g/8.4g_{portion}$	%RDI[3]	$g/8.4g_{portion}$	%RDI[3]	$g/8.4g_{portion}$	%RDI[3]	$g/8.4g_{portion}$	%RDI[3]	$g/8.4g_{portion}$	%RDI[3]
Protein	50	2.00	4.0	2.23	4.5	1.36	2.7	1.58	3.8	1.42	3.4
Total sugars	270	4.59	1.7	4.43	1.6	5.45	2.0	5.15	2.3	5.01	2.2
Fat	70	0.29	0.4	0.19	0.3	0.29	0.4	0.24	0.4	0.27	0.5
	mg/day[2]	$mg/8.4g_{portion}$	%RDI[3]	$mg/8.4g_{portion}$	%RDI[3]	$mg/8.4g_{portion}$	%RDI[3]	$mg/8.4g_{portion}$	%RDI[3]	$mg/8.4g_{portion}$	%RDI[3]
Calcium	800	1.42	0.2	1.42	0.2	1.52	0.2	1.46	0.2	1.90	0.2
Potassium	2000	240.85	12.0	220.78	11.0	220.70	11.0	242.05	12.1	313.89	15.7
Magnesium	375	12.65	3.4	11.41	3.0	11.20	3.0	7.97	2.1	13.21	3.5
Phosphorus	700	70.15	10.0	88.97	12.7	82.21	11.7	64.03	9.2	91.50	13.1
Iron	14	0.78	5.6	1.96	14.0	0.35	2.5	0.59	4.2	2.33	16.7
Zinc	10	0.88	8.8	1.30	13.0	0.72	7.2	0.55	5.5	1.02	10.2
Copper	1	0.10	9.6	0.21	20.8	0.07	7.0	0.16	16.0	0.23	23.0
Manganese	2	0.06	3.1	0.08	4.2	0.08	4.0	0.13	6.7	0.15	7.40

[1]% Total Sugars (%) = Organic matter (%) – Total Protein (%) - Total Fat (%); a-f, in a raw: Different letters indicate significant differences (p<0.05) between species;
[2]WHO, 2004; [3]Based on a serving size of 84 g fresh mushroom (O'Neil et al., 2013) equivalent to 8.4 g portion of dry mushroom.

Pleurotus citrinopileatus var. *cornucopiae* had a similar proximate composition to *P. salmoneo stramineus* differing to *P. eryngii* (Table 4.2). A similar proximate composition characterized *Ph. nameko* and *H. erinaceus* with 16.8-19.2, 59.6-61.2 and 2.9-3.2 g/100$g_{dry\ mushroom}$ of total protein, total sugar and total fat content, respectively.

It is known that mushroom proximate composition may be affected by several factors, such as species, development stage, maturity of fruiting body, mushroom sampled part, level of nitrogen available (Colak et al., 2009) as well as by substrate composition and cultivation procedures (La Guardia et al., 2005). These results demonstrate such interspecies variability and corroborate the importance of cultivation optimization and standardization to increase yield and nutritional value. Protein content in the five similarly cultivated mushrooms ranged between 16.2-26.6 g/100$g_{dry\ mushroom}$ for *Pleurotus* spp. and 17-19 g/100$g_{dry\ mushroom}$ for *Ph. nameko* and *H. erinaceus*, respectively (Table 4.2). Lower protein contents (8.5-19.7% dried mushroom) was reported by Akyüz & Kirbağ (2010) who studied the effect of various agro-wastes (wheat straw, wheat strawcotton stalk and rice bran) on the nutritive value of cultivated *P. eryngii* var. *ferulae*. La Guardia et al. (2005) reported a proximate composition with higher levels of protein (26.6 g/100 $g_{dry\ mushroom}$), similar to those reported for *P. salmoneo stramineus* herein, for *P. eryngii* var. *eryngii* cultivated in wheat straw and sugar beet based substrate. Reported values of protein content for *P. citrinopileatus* are lower than those reported herein and ranged between 9.2-17.2 g/100$g_{dry\ mushroom}$ when cultivated on substrate based on the sludge of handmade paper and cardboard industrial waste used alone or in combination with wheat straw (Kulshreshtha et al., 2013). A value of 15.6 g/100$g_{dry\ mushroom}$ for total protein was reported by Guo et al. (2007) for cultivated *P. djamor* (former name of *P. salmoneo stramineus*) purchased in a local supermarket in Guangzhou city, China. According to Khan & Tania, (2012) *Pleurotus* species are recognized as good source of protein with values ranging from 11 to 42 g/100$g_{dry\ mushroom}$, fitting well the results reported for the 3 species included in this study.

Low total fat contents (2.3-3.5 g/100$g_{dry\ mushroom}$) and high total sugar contents (52.7-64.9 g/100$g_{dry\ mushroom}$) characterize, in general, the cultivated mushrooms analysed similarly to those found in the literature for cultivated *P. eryngii* var. ferulae (Akyüz & Kirbağ, 2010). Lower total fat content was reported for *P. citrinopileatus* cultivated in different substrate (0.13 to 0.46 g/ 100$g_{dry\ mushroom}$; Kulshreshtha et al., 2013) and for *P. djamor* (1.65 g/ 100$g_{dry\ mushroom}$; Guo et al., 2007). In turn, La Guardia et al. (2005) reported much higher values for fat content (8.6 g/100$g_{dry\ mushroom}$) for *P. eryngii* var. *eryngii* cultivated in wheat straw and sugar beet based substrate; other reported fat values for *Pleurotus* species ranged between 0.5 and 8 g/100$g_{dry\ mushroom}$ (Khan & Tania, 2012). The characteristic low fat content found in mushrooms coupled to its unsaturated nature provided by the predominating linoleic and oleic free fatty acids (Kalač, 2013) is of great dietary importance.

Mushrooms are a good source of carbohydrates. Values ranging between 51.4-59.9 g/100$g_{dry\ mushroom}$ were reported by La Guardia et al. (2005) and by Guo et al. (2007) for *P. eryngii* var. *eryngii* and for *P. djamor*, respectively. According to Kalač (2013) the carbohydrate content constitutes about one-half of mushroom dry matter being present mainly as polysaccharides and glycoproteins

making them suitable for incorporation into low-calorie diets; 36-60 g/100$g_{dry\ mushroom}$ of carbohydrates have been reported for *Pleurotus* species (Khan & Tania, 2012) which are comparable to the data in Table 4.2.

Comparison between proximate composition data for cultivated *Ph. nameko* and *H. erinaceus* and published data was not possible because to the best of my knowledge the only existing characterization studies targeted wild-growing *H. erinaceus*. Mau et al. (2001) reported carbohydrate, moisture, crude protein, fat and ash content values of 57.0, 4.3, 22.3, 3.5 and 9.4 % of air dried weight for wild-growing *H. erinaceus*, respectively.

Edible mushrooms have been reported as a source of phenolic compounds with antioxidant properties (Preeti et al., 2012). According to Preeti et al. (2012) natural phenolic compounds are produced and accumulated with end products ranging from simple molecules (phenolic acids) to highly polymerised compounds (tannins). Among the 5 edible mushroom species tested, the Pleurotaceae family revealed great variability englobing the highest (1140 µg cathecol equiv/$g_{dry\ mushroom}$ found in *P. citrinopileatus* var. *cornucopiae*) and the lowest (464 µg cathecol equiv/$g_{dry\ mushroom}$ found in *P. salmoneo stramineus*) total phenolic contents. This trend somewhat contrasts with those reported by Mishra et al. (2013) who determined the total phenolic content in mushroom mycelium of the same 3 species, i.e. *Pleurotus eryngii, P. djamor* and of *P. citrinopileatus* but grown in malt extract; higher values were observed for mycelium of *P. eryngii*, followed by *P. djamor* and *P. citrinopileatus*, respectively.

Apparent higher phenolic content was reported by Yildirim et al. (2012) for wild-grown edible *P. eryngii* (29 to 32 mg of gallic acid equiv/$g_{dry\ mushroom}$) collected from different regions of Tunceli (Turkey). However direct comparison will not be appropriate given their different origin: cultivated *vs* wild-growing mushrooms. In terms of cultivated *Pleurotus* sp., Reis et al. (2012b) reported 7.14 mg of gallic acid equiv/$g_{methanolic\ extract}$ from *P. eryngii* mushrooms obtained in local supermarkets (Bragança, Northeast Portugal).

Ash content was quite variable and statistically different among the different species, ranging from 5.99 to 8.40 g/100$g_{dry\ mushroom}$ in *P. eryngii* and in *Ph. nameko* (Table 4.2). No statistical differences were observed between *Pleurotus spp.* for ash content.

4.3.2. Fatty acids profile of cultivated mushrooms

From the analysis of data in Table 4.3, it was found that total FA concentration ranged from 22.75 to 14.12 µg/$mg_{dry\ mushroom}$ in *P. eryngii* and in *P. salmoneo stramineus* (p<0.05). All samples showed concentrations of linoleic acid (C18:2 c9 c12) above 30 g FA/100g_{fat}, indicating that this is main FA in the composition for *P. salmoneo stramineus* and *P. citrinopileatus* var *cornucopiae* (69.09-78.33 g FA/100g_{fat}). The other mushroom species had significantly lower values especially in *H. erinaceus* (38.69 g FA/100g_{fat}). The FA composition of the mushroom species was characterized by much higher content of mono (MUFA) and polyunsaturated FA (PUFA) than of saturated FA

Table 4.3. Fatty acid composition (g FA/100g_{fat}) and total content (µg FA/mg_{DM}) of cultivated edible mushroom species.

	Pleurotus citrinopileatus var. cornucopiae		*Pleurotus salmoneo stramineus*		*Pleurotus eryngii*		*Hericium erinaceus*		*Pholiota nameko*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14	0.17 b	0.02	0.18 b	0.01	0.26 a	0.01	0.24 ab	0.01	0.16 b	0.02
C15	2.31 a	0.02	0.82 d	0.07	1.27 b	0.02	1.44 c	0.03	1.88 a	0.05
C16	16.52 b	0.07	12.45 c	0.04	12.49 c	0.03	18.60 a	0.02	10.29 b	0.66
C16:1 c7 n9	<LOD c	—	<LOD c	—	0.10 b	0.02	0.17 a	0.01	0.06 b	0.01
C16:1 c9	0.22 b	0.02	0.23 b	0.01	0.26 b	0.04	0.39 a	0.01	0.22 b	0.02
C17i	0.20 b	0.04	0.36 a	0.02	0.14 b	0.01	0.11 b	0.02	0.19 b	0.02
C16:2 c9 t12	<LOD c	—	<LOD c	—	0.04 b	0.01	0.38 a	<0.01	<LOD c	—
C17	0.32 c	<0.01	0.58 a	0.05	0.17 b	<0.01	0.49 a	0.02	0.21 bc	0.02
C17:1 c9 n6	<LOD b	—	<LOD b	—	<LOD b	—	0.08 a	0.01	<LOD b	—
C17:1 c10	<LOD d	—	0.12 b	0.04	0.07 c	<0.01	0.14 b	<0.01	0.15 a	0.02
C18	1.79 b	0.01	1.85 b	0.02	1.66 c	0.01	6.15 a	0.04	1.64 c	0.03
C18:1 t4+t5	<LOD c	—	0.20 a	0.01	0.09 b	<0.01	0.09 b	0.02	0.14 b	0.01
C18:1 t9 n9	<LOD c	—	<LOD c	—	0.05 b	0.01	0.07 b	0.01	0.13 a	0.03
C18:1 c9 n9	5.47 c	0.05	3.71 c	0.01	27.13 a	0.01	27.36 a	0.09	17.82 b	0.01
C18:1 c11	0.63 d	0.04	0.30 e	0.01	0.82 c	0.01	4.58 a	0.12	3.51 b	0.03
C18:2 c9 t12 n6	0.09 a	<0.01	0.11 a	0.02	0.13 a	0.01	0.09 a	0.01	0.12 a	0.02
C18:2 t9 c12 n6	<LOD b	—	0.07 a	0.01	<LOD b	—	0.04 a	<0.01	0.06 a	<0.01
C18:2 c9 c12 n6	69.09 b	0.19	78.33 a	0.10	53.90 d	0.05	38.69 e	0.15	62.22 c	0.05
C18:3 c9 c12 c15 n3	<LOD b	—	<LOD b	—	0.09 a	<0.01	0.05 a	<0.01	0.08 a	0.04
C20	<LOD c	—	<LOD c	—	0.11 b	0.01	0.11 b	0.02	0.21 a	0.01
C20:1 c11 n9	<LOD c	—	<LOD c	—	0.13 a	0.02	0.05 b	0.01	0.11 a	0.03
C20:3 c11 c14 c17 n3	2.74 a	0.09	0.42 b	0.01	0.43 b	0.01	0.24 c	0.01	0.08 d	<0.01
C24	0.46 a	0.01	0.28 b	0.04	0.27 b	0.01	0.42 ab	0.01	0.35 b	<0.01
C24:1 c15 n3	<LOD b	—	<LOD b	—	0.39 a	0.02	<LOD b	—	0.37 a	0.02
SFA (%)	21.76 b	0.01	16.51 c	0.06	16.36 c	0.02	27.56 a	0.02	14.93 d	0.04
MUFA (%)	6.32 d	0.11	4.56 e	0.07	29.04 b	0.06	32.94 a	0.01	22.51 c	0.04
PUFA (%)	71.93 b	0.10	78.93 a	0.13	54.59 d	0.08	39.50 e	0.02	62.56 c	0.01
µg FA/mg_{DM}	14.14 c	0.32	14.12 c	0.50	22.75 a	0.57	19.37 b	0.36	17.20 b	0.62

Data expressed as mean (Mean; n=3) and standard deviation (SD). ai: anteiso. Phy: Phytanic acid. AA: Arachidonic acid. c/t: cis/trans double bond. SFA/MUFA/PUFA: Percentage of total of saturated/monounsaturated/polyunsaturated fatty acids; n3, n6 and n9: Omega-3, -6 and -9 fatty acids. DM: dry matter. a-f: in a row, significant differences among seaweed species.

(SFA). The SFA distribution was characterized by palmitic (C16) and stearic acids. (C18). Higher content was observed in *H. erinaceus* and in *P. citrinopileatus* var *cornucopiae* (16.52-18.60 g FA/100g_{fat}) whereas values between 10.3-12.5 g FA/100g_{fat} were observed for the other mushroom species. For C18 the highest amount for this FA was observed in *H. erinaceus* (6.15 g FA/100g_{fat}; p<0.05) which was 3.3 to 3.7 times higher compared to the other mushroom species (1.64-1.85 g FA/100g_{fat}).

Interestingly, in *P. salmoneo stramineus* and *P. citrinopileatus* var *cornucopiae* the concentration of linoleic acid was higher than 65% but the concentration of oleic acid (C18:1 c9) was the lowest (3.7-5.5 g FA/100g_{fat}). For the other mushroom species this ratio was lower and more variable (Table 4.3).

The FA composition of edible mushrooms can comprise from butyric acid (C4) to docosahexanoic acid (C22:6) depending on the mushroom species but in general C16, C18:1 c9 and C18:2 c9 c12 are among the main FA (Ergönül et al., 2013). Previous studies reporting the FA composition of wild *P. eryngii* or obtained from local supermarkets in Portugal, showed that SFA content were 25.8-17.4%, MUFA were 49-13.1% and PUFA were 69.4-25.2% (Reis et al., 2012a; Reis et al., 2014). Such variation was associated to the values of palmitic (14.9-12.8%), oleic (47.5-12.3%) and linoleic acids (68.8-24.7%). However the detailed profile was solely focused on the mentioned FA as well as on the stearic and linolenic acids.

To my knowledge this is the first study describing the FA composition of the edible *P. salmoneo stramineus, P. citrinopileatus* var *cornucopiae*, *Ph. nameko* and *H. erinaceus* and therefore this work brings valuable data about the nutritional evaluation of such mushrooms species.

4.3.3. Elemental composition of cultivated mushrooms

Cultivated mushrooms have been reported as a good source of minerals, and may contain Ca, Mg, Na, K, P, Cu, Fe, Mn, Zn (Cheung, 2008). In this study, the species analysed revealed to be good sources of macroelements such as K, Mg, and P, where K stood out as the most predominant macroelement with values ranging between 26274-37368 mg/$Kg_{dry\ mushroom}$ followed by P (7623-10892 mg/$Kg_{dry\ mushroom}$) and Mg (949-1572 mg/$Kg_{dry\ mushroom}$) (Figure 4.1a-f). Their combined percentage was about 98-99% where K represented 6-76%, followed by P with 20-27% and Mg with 2.5-3.9%. Higher contents, but not statistically different (p>0.05), of K, P and Mg were observed for both *P. eryngii* and *P. salmoneo stramineus*. Higher content of K was observed in *Ph. nameko* (p<0.05) in comparison to *H. erinaceus*. Potassium and Mg were also the predominant elements in other cultivated *Pleurotus* spp. For example, Lee et al. (2009) reported 26273 and 1233 mg/$Kg_{dry\ mushroom}$ for K and Mg for *P. eryngii* cultivated in complex substrate based on pine sawdust, corncob and rice bran with beet pulp (sawdust and beet pulp are two common organic substrates also used to cultivate *P. eryngii* - Table 4.1). Akyüz & Kirbağ (2010) reported 14.3-18.8 mg/$g_{dry\ mushroom}$ for K for *P. eryngii* var. *ferulae*. Phosphorus content was not reported in both

studies. Guo et al. (2007) also reported abundance of these macroelements in *P. djamor*: 1.21, 7.57 and 12.3 mg/$g_{dry\ mushroom}$ for Mg, P and K. According to Kalač (2013) K is highly accumulated in mushroom fruit bodies, reaching 20-40-fold higher values than in the substrate.

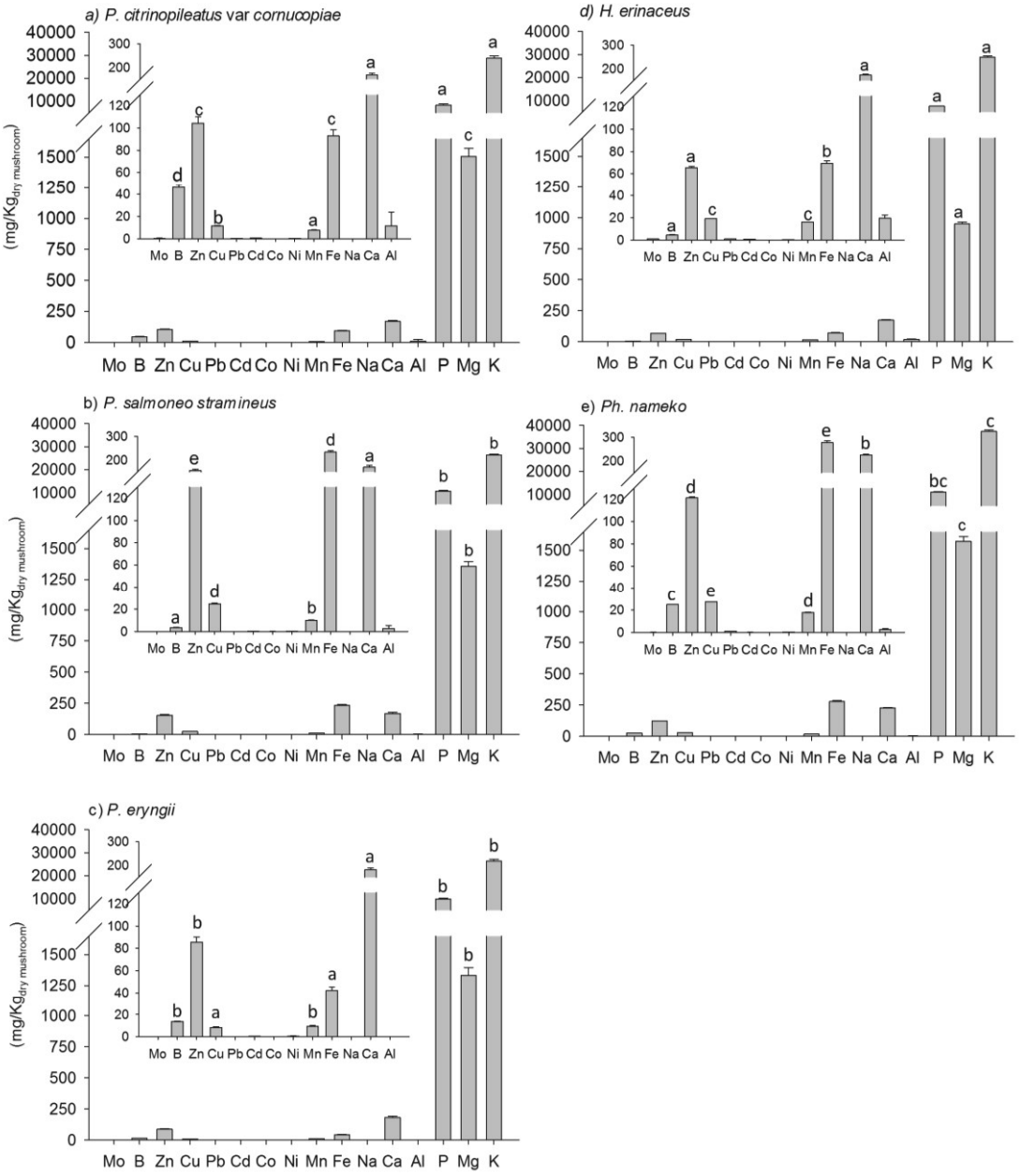

Figure 4.1. Elements content (mg/$kg_{dry\ mushroom}$) in the different cultivated mushroom species: a) *P. citrinopileatus* var. *cornucopiae*; b) *P. salmoneo stramineus*; c) *P. eryngii*; d) *H. erinaceus*; e) *Ph. nameko*. For each element, different letters indicate significant differences (p<0.05) between mushroom species.

Among the remaining macroelements, Ca was found at rather low levels with values ranging from 170-226 mg/$Kg_{dry\ mushroom}$ and sodium (Na) was not detected above limit of detection.

According to Cashman (2002), Ca is the specific nutrient most important for preventing and treating osteoporosis. This finding coupled to the non-detection of Na may be considered as good advantages from a nutritional aspect.

Statistically higher content of Ca was observed in *Ph. nameko* (p<0.05) in comparison to other mushrooms tested, but the content of Ca was not statistically different among the three cultivated *Pleurotus* spp. and *H. erinaceus*. Variable content of Ca and Na were reported in the literature for cultivated *Pleurotus* spp. For example higher content of Ca (120-700 mg/$kg_{dry\ mushroom}$) in comparison to Na (100-307 mg/$kg_{dry\ mushroom}$) were reported in *P. eryngii* var. *ferulae* (Akyüz & Kirbağ, 2010) but the opposite was observed in other cultivated *P. eryngii:* 8 and 147 mg/100$g_{mushroom}$ reported by La Guardia et al. (2005) and 162.5 and 253.6 mg/$kg_{dry\ mushroom}$ reported by Lee et al. (2009) for Ca and Na, respectively. According to Lee et al. (2009) their reported Ca content in the mushroom fruit bodies was very low despite high concentrations in the substrate. The authors suggest that either Ca was present in the substrate in a less bioavailable form or mushrooms do not have efficient Ca uptake channels.

The most predominant observed microelements were Zn and Fe in the 5 cultivated mushroom species analysed (Figure 4.1a-f). According to Khan & Tania (2012) Fe and Zn are the most abundant elements among the trace minerals in mushrooms. In particular, Fe content was high in *P. salmoneo stramineus* and in *Ph. nameko* (233 and 278 mg/$Kg_{dry\ mushroom}$) whereas Zn was high in *P. salmoneo stramineus, P. citrinopileatus* var. *cornucopiae* and in *Ph. nameko* (155, 104 and 121 mg/$Kg_{dry\ mushroom}$). The reported values were, in general, higher than those reported by Gençcelep et al. (2009) for various edible mushrooms collected from the Erzurum region of Turkey. Lee et al. (2009) reported 39.0 mg/$Kg_{dry\ mushroom}$ for Fe and 52.2 mg/$Kg_{dry\ mushroom}$ for Zn for cultivated *P. eryngii* whereas Akyüz & Kirbağ (2010) reported 519-620 mg/$kg_{dry\ mushroom}$ for Fe, 40.5-102.5 mg/$kg_{dry\ mushroom}$ for Zn for *P. eryngii* var. *ferulae.* Iron is known to be essential for cellular energy and metabolism (Jankowska et al., 2013) and its deficiency is associated to anaemia which affect adversely patients with chronic heart disease (Comín-Colet et al., 2013). Zinc is present in all organs, tissues, fluids, and secretions participating in all major biochemical pathways playing multiple roles in the perpetuation of genetic material (Brown et al., 2004). Zinc deficiency may be associated to adverse outcomes of pregnancy (King, 2000), sickle cell disease (Prasad, 2002) or metabolic syndrome and diabetes (Miao et al., 2013). Among the remaining trace elements it is worthwhile referring Cu contents that were clearly higher in *P. salmoneo stramineus* and in *Ph. nameko* (24.7 and 27.3 mg/$Kg_{dry\ mushroom}$) than in the other cultivated species analyzed. According to Khan & Tania (2012) the inclusion of *Pleurotus* spp. mushrooms in the diet could help to minimize Fe, Zn, Cu and other micronutrients deficiencies, but their bioavailability still have to be tested in animal and human studies since contradictory results still do persist in literature.

In general, the five cultivated mushrooms studied were of important nutritional value not only due to their proximate composition but especially due to their elemental composition. Taking current trends into account, studied mushrooms offer a wide array of nutrients, at concentrations that may meet with nutritional requirements, particularly in what concerns elements and, in some

cases, may even allow for application of nutritional claims. Data on daily intake of mushrooms are unavailable for the Portuguese population and according to O'Neil et al. (2013) mushroom intake data are sparse. Considering a serving size of 84 g of fresh mushroom, reported by O'Neil et al. (2013) based on FDA food labelling information (2013) the possible contribution of analysed mushrooms to daily nutritional requirements was calculated (Table 4.2). Whereas such daily intake contributes with a small fraction to protein, carbohydrate and fat requirements, in terms of microelements some of the mushrooms contribute over 15% (minimum requirement for nutritional claim) of the recommended daily intakes (RDIs). Variable contribution to the RDI for the elements is observable in Table 4.2. In terms of macroelements all species can be considered good contributors to RDI of K, especially *Ph. nameko* with values as high as 15.7%. In terms of P, values between 9.2 and 13.1% RDI are reported with higher values found in *Ph. nameko*. Lower Ca and Mg suppliers characterized the cultivated species. In terms of microelements, interesting values of contribution to Fe, Cu and Zn RDI was observed in *P. salmoneo* and *Ph. nameko* with values ranging between 10 to 23%. Lower values were, in general, found for Mn.

According to O'Neil et al. (2013) mushroom consumption is associated with a better nutrient profile and higher diet quality. For example higher intakes of Cu and K are reported by these authors for mushroom consumers than non-consumers. The recommendations by O'Neil et al. (2013) go further and state that mushroom consumption should be encouraged by health professionals. In a meta-analysis on mushroom intake and its relation to the reduction of breast cancer, Li et al. (2014) concluded that greater edible mushroom consumption may be associated with a lower risk of breast cancer.

4.3.4. FTIR-ATR characterization

Mushrooms are rich in polysaccharides followed by protein content with low fat content (Table 4.2) yet a more specific characterization of these macrostructures is a further asset to establish structure-function relationships. Several authors (Zhao et al., 2006ab) describe the chemical characteristic features of specific regions of FTIR spectra for mushrooms truffles (Tuberaceae) and for different *Amanita* species, according to:

i) 4000-1800 cm^{-1} with a prominent broad band centred around 3300 cm^{-1}, that could be assigned to O-H and C-H stretching vibrations and two sharper bands around 2900-2880 cm^{-1} assigned to CH_2 and CH_3 stretching of fatty acids from the cell wall;
ii) 1800-1500 cm^{-1} with two major bands around 1650 and 1560 cm^{-1} assigned to amide I and amide II of proteins; a band around 1740 cm^{-1} that could correspond to carbonyl stretching vibration of alkyl-esters indicating the presence of oil;
iii) 1500-750 cm^{-1} region associated with vibrations of proteins, lipids but also polysaccharides - 1077 and 1042 cm^{-1} have been assigned to C-O stretching of polysaccharides;

iv) 950-750 cm^{-1} region that has been associated with identification of anomeric configuration of polysaccharides - 890 cm^{-1} band has been assigned to β-glycosides and 860-810 cm^{-1} for α-glycosides. This information is equally supported by Mohaček-Grošev et al. (2001) studies who used vibrational spectroscopy to characterize several wild growing mushroom species.

In figure 4.2a-b, the 4 specific regions are observable for the 5 mushroom species. At first sight there seems to be little qualitative difference between spectra of the 3 cultivated *Pleurotus* species (Figure 4.2.a); apparent differences are more evident in *Ph. nameko* spectrum especially in the 1500-750 cm^{-1} region which is associated with vibrations of proteins, lipids but also polysaccharides (Figure 4.2.b). Evidence of presence of proteins (due to 1650 and 1560 cm^{-1} bands), fatty acids (due to two sharper bands around 2900-2880 cm^{-1}) and polysaccharides (bands in 1500-750 cm^{-1} region) are easily perceived in the 5 cultivated mushroom species spectra taking into account the band assignments by Zhao et al. (2006b) and Mohaček-Grošev et al. (2001) According to Liu et al. (2006) the region between 750-1200 cm^{-1} could serve as fingerprints to discriminate mushrooms whereas according to Mohaček-Grošev et al. (2001) the spectral region between 1200-1000 cm^{-1} could serve as an indicator of mushroom genus.

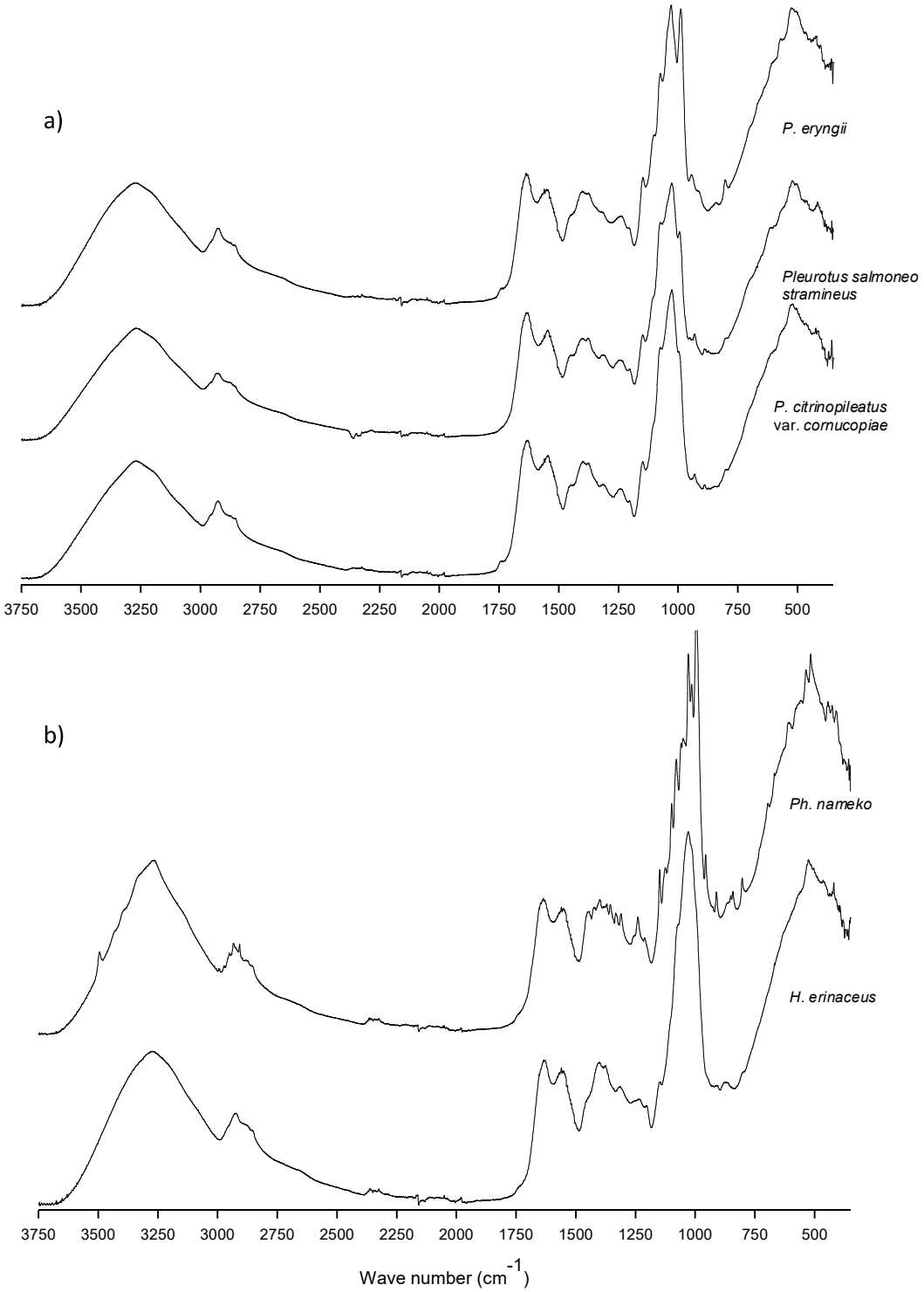

Figure 4.2. FTIR-ATR spectra of the five cultivated mushroom species: a) *P. eryngii*, *P. salmoneo stramineus* and *P. citrinopileatus* var. *cornucopiae*; b) *Ph. nameko* and *H. erinaceus*.

In figures 4.3 and 4.4, the 1500-750 cm^{-1} region is amplified enabling a more detailed observation of the bands present in this region. Characteristic bands found in the 5 spectra are 1400, 1375, 1315, 1237-1240, 1204-1212, 1148, 1074-1079, 1026-1029, 995-988, 890-910 and

801-803 cm^{-1}. The majority coincide with main observed bands in the 5 Amanitas species and the 5 truffles studied (Zhao et al., 2006ab). According to Gonzaga et al. (2005) 1028 cm^{-1} is assigned to C-O stretching, 1074 cm^{-1} to anomeric C_1H group vibration and 1165 to C-O-C stretching of glycosidic structures. Protein patterns have been associated with characteristic absorption at 1654, 1544, 1409 and 1242 cm^{-1}; a similar pattern was found in the 5 cultivated mushroom species studied which according to Gonzaga et al. (2005) may be evidence of the presence of a glucan-protein complex.

Although bands between 950-750 are very weak they are considered important for the identification of anomeric configuration of polysaccharides (Barbosa et al., 2003; Mohaček-Grošev et al., 2001). According to Zhao et al. (2006ab) β-glucan and chitosan standards with β-glycosidic linkage presented bands at 889 and 897 cm^{-1}, respectively, whereas α-glycosidic linkage typical in a standard starch presented a characteristic band at 858 cm^{-1}.

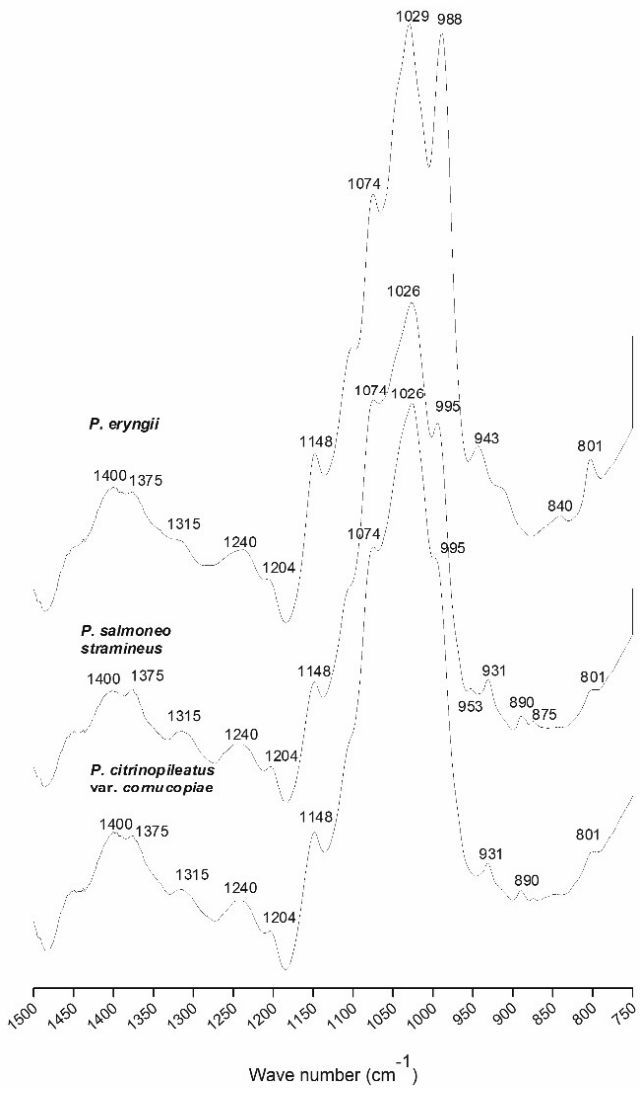

Figure 4.3. FTIR-ATR spectra of the three Pleurotus sp. (*P. eryngii*, *P. salmoneo stramineus* and *P. citrinopileatus* var. *cornucopiae*) mushrooms between 1500 and 750 cm^{-1}.

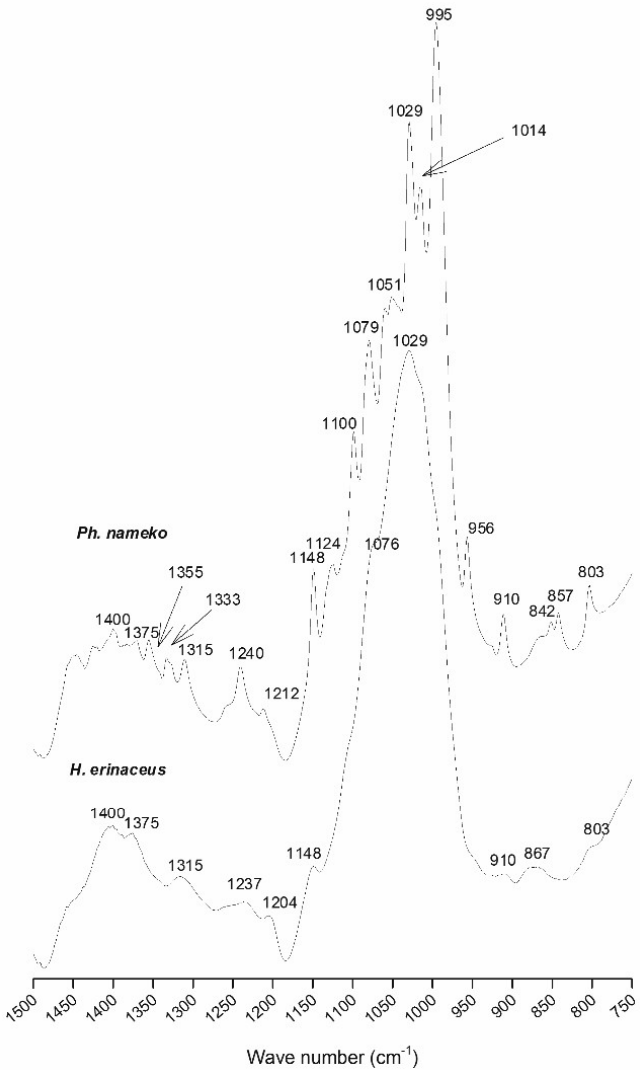

Figure 4.4. FTIR-ATR spectra of *Ph. nameko* and *H. erinaceus* between 1500 and 750 cm^{-1}.

According to Barbosa et al. (2003) bands at 890 and 1370 $cm^{-1}$are typical of (1→3)-β-glucans and of β-glucans, respectively. In *P. citrinopileatus* var. *cornucopiae* and *P. salmoneo stramineus* spectra (Figure 4.3) as well as in *Ph. nameko* and *H. erinaceus* spectra (Figure 4.4), two weak bands at 890-910 cm^{-1} and 801-803 cm^{-1} were observable; such could indicate that both α- and β-glycosidic linkages exist in these mushroom species. In addition, the detection of the band at 1375 cm-^{1} in the five cultivated mushroom species suggests the existence of β-glucans. According to Wasser (2002) mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1→3), (1→6)-β-glucans and (1→3)-α-glucans but also as heteroglucans; in this case, side chains contain glucuronic acid, xylose, galactose, mannose, arabinose, or ribose as a main component or in different combinations.

Possible discriminant bands between the 3 cultivated *Pleurotus* species are the bands at 943 and 840 cm^{-1} only present in *P. eryngii*, 953 and 875 cm^{-1} only present in *P. salmoneo stramineus*, whereas the band at 931 cm^{-1} is visible in *P. salmoneo stramineus* and *P. citrinopileatus* var. *cornucopiae* (Figure 4.3). In turn, possible discriminant bands for *Ph. nameko* are observable in the 1500-1000 cm^{-1} region such as 1124, 1100, 1014 cm^{-1} and in the 950-750 cm^{-1} region such as 956, 842 and 851 cm^{-1}. The *Ph. nameko* spectrum is characterized by more and diverse distinct bands than the other 4 cultivated mushroom species including in the 1500-1200 cm^{-1} region. A broad weak band around 867 cm^{-1} appears to be discriminative for *H. erinaceus* (Figure 4.4).

Zhao et al. (2006a,b) suggested several absorption ratios to discriminate between mushroom species. In accordance, the absorption ratios $A_{1029/1148}$, $A_{1029/1074}$ were calculated for each cultivated mushroom species. The ratio $A_{1029/1148}$ did not reveal to be discriminant (1.29-1.10), which is not the case for the $A_{1029/1074}$ ratio where clear differences were detected; calculated values were 1.24, 1.18, 0.41, 0.41 and 0.38 for *P. eryngii*, *P. salmoneo stramineus*, *P. citrinopileatus* var. *cornucopiae, H. erinaceus* and *Ph. nameko*, respectively.

4.4. Conclusions

The chemical composition of five cultivated edible mushrooms in terms of proximate composition, fatty acids profile and elemental composition showed significant differences among the different species, emphasizing the nutritional potential of the five different cultivated edible mushrooms to be consumed in a healthy diet. High contents in proteins and polysaccharides associated with low content of fat, which profile is characterized by higher concentration in mono and polyunsaturated FA than in saturated FA, being also interesting sources of phenolic compounds as well as of some macro and micronutrients highlights its potential as healthy food. According to FTIR-ATR spectra, the presence of β-glucans, α-glucans and glucan-protein complexes are among main representative polysaccharides in the five species. The presence of these polysaccharides further upholds the interest in exploring these mushrooms for applications in health-related fields, for example, drug or nutraceutical delivery approaches.

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Chapter 5

Impact of enzyme and ultrasound assisted extraction methods on biological properties of *Sargassum muticum* (brown seaweed), *Osmundea pinnatifida* (red seaweed) and *Codium tomentosum* (green seaweed)

5.1. Introduction

As already discussed seaweeds of great taxonomic diversity are still an untapped source of diverse chemical compounds of interest including nutrients such as polysaccharides, proteins, minerals, vitamins and dietary fibres (Holdt & Kraan, 2011). The ingestion of such seaweed compounds has the advantage of being of natural origin affecting positively the health of individuals or even reducing the risk of certain pathologies (Ibañez & Cifuentes, 2013). Important biological properties such as antioxidant, antibacterial, anticoagulant and antitumor activities have been associated to seaweeds all of which represent added value for alternative food species (Pangestuti & Kim, 2011; El Gamal, 2010).

Extraction and isolation of compounds of interest from seaweeds, able to be ingested or used for food purposes, need to rely upon compatible methods with economically viable yields. Water-based extraction is food compatible, non-expensive and environment friendly but has low selectivity with low extraction efficiency (Herrero et al., 2006; Heo et al., 2003). However seaweeds are known to have a chemical and structural heterogeneous rigid wall which poses limitations to efficient extraction of the intracellular and wall compounds (Jeon et al., 2012). Considering the limitations of water-based extraction (low selectivity and low extraction yields), yet wishing to target efficient food compatible methods, the main objective of the study in this chapter was to obtain extracts rich in bioactive compounds using alternative water-based approaches such as enzyme assisted extraction (EAE) and ultrasound assisted extraction (UAE) on representative red, brown and green species from the six species studied and characterized in chapter 3, namely on *Sargassum muticum* as well as on of the some of which less studied such as *Osmundea pinnatifida* and *Codium tomentosum*. Hence, this study was based on 3 steps: i) performance of different extraction modes and evaluation of respective yield; ii) chemical characterization of each extract obtained from each of the three different seaweeds and, iii) evaluation of the biological potential of the extracts in terms of antioxidant, anti-diabetic and prebiotic activities.

Seaweeds such as *Sargassum* species (Phaeophyceae*,* brown seaweed), have been found to be good sources of dietary fibre and of phenolic compounds and carotenoids with antioxidant activity, all of which play important roles in the prevention of intestinal and neurodegenerative diseases, respectively (Tanniou et al., 2014; Je et al., 2009; Chandini et al., 2008). Several other compounds able to scavenge and/or neutralize oxidative compounds (antioxidant activity) have been isolated from other seaweeds including phenolic compounds, sulphated polysaccharides or proteins (Farvin et al., 2013; Heo et al., 2005).

Prebiotics are defined as non-digestible compounds mainly of carbohydrate nature that selectively enhance the activity and viability of beneficial bacteria in the intestine, by providing these with fermentable substrates that lead to the production of short chain fatty acids (Al-Sheraji et al., 2013). They have been associated with several health benefits in the large intestine such as modulation of beneficial gut bacteria, hypocholesterolemic effect, reduction of cancer and obesity risks or increase of the bioavailability and uptake of some minerals (Ca and Mg) (Al-Sheraji et al.,

2013). According to Zaporozhets et al. (2014), the prebiotic activity of extracts or of polysaccharides from marine seaweeds, combined with a broad spectrum of biological properties, evidences great potential for their use as functional nutrition enabling modulation of intestinal microflora and of gastrointestinal tract (GIT) inflammations as well as to normalize the immune system.

Inhibitory compounds of α-glucosidase or α-amylase with anti-diabetic activity are of much interest since by acting as competitive inhibitors of the enzymes they inhibit the hydrolysis of oligo-, tri- and disaccharides to glucose and other monosaccharides in the small intestine and thereby delay postprandial glucose absorption lowering blood glucose (Ali et al., 2006). Inhibition of the carbohydrate hydrolysing enzymes have been reported for different extracts of red and green seaweeds (Senthil et al., 2013).

To my knowledge this is the first study applying water-based extraction through combined EAE and UAE extraction technologies in *S. muticum*, *O. pinnatifida* and *C. tomentosum* seaweeds and there are no reports regarding the prebiotic and anti-diabetic potential of water-based enzyme-assisted extracts for these studied species.

5.2. Material and methods

5.2.1. Selected seaweeds species

As previously stated, three species of seaweeds were selected from the six studied in chapter 3. The species selected were specimens of the red alga (Rhodophyta, Florideophyceae) *Osmundea pinnatifida* (Ceramiales) Rhodomelaceae family, the brown alga (Heterokontophyta, Phaeophyceae) *Sargassum muticum* (Fucales) Sargassaceae family and of the green alga (Chlorophyta, Ulvophyceae) *Codium tomentosum* (Bryopsidales) Codiaceae family, which were harvested in April 2012 from Buarcos bay (Figueira da Foz, Portugal). The classification of seaweeds was based on *AlgaeBase* (Guiry & Guiry, 2013). The seaweeds were first washed with running tap water and then with deionised water and then dried in an oven at 60 ºC. The dried samples of seaweeds were milled to less than 1.0 mm particle size.

5.2.2. Ultrasound and enzymatic-assisted extraction

Ultrasound assisted extraction and EAE as well as hot water extraction (HWE) were performed on the three different seaweeds (*S. muticum, O. pinnatifida* and *C. tomentosum*). Two carbohydrate degrading enzymes (Viscozyme L, Cellulase) and two proteases (Alcalase, Flavourzyme) were tested for EAE. All extraction procedures were conducted in triplicate.

For HWE, 2 g of dried seaweed were dispersed in 50 mL of deionized water and incubated in an agitated water bath at 50 ºC for 24h in the darkness. The aqueous solution (4% dw) was then centrifuged at 5000 g for 10 min at 4 ºC (centrifuge Medifriger BL-S, JP Selecta, Spain) and the

supernatant filtered with glass filter funnel (porosity 1) and the extract frozen at -80 ºC until lyophilisation.

UAE and EAE were performed according to Huang et al. (2010) and Wang et al. (2010) respectively, with modifications. The UAE extracts were prepared based on HWE procedure except that after 24h at 50 ºC, a ultrasound extraction was carried out in a water bath ultrasonicator (Ultrasonik 57H Ney, 400W, 50/60 Hz) for 60 min (sonicate for 10 minutes and pause for 2 minutes) at 50 ºC. The resultant aqueous solution was then centrifuged, filtered and frozen according to HWE procedures.

For the EAE extracts, the same amount of seaweed (2g) was dispersed in 50 mL and incubated in an agitated water bath for 10 min. After adjusting pH to specific enzyme optimum conditions (Table 5.1), 100 mg of enzyme was added and incubated for enzymatic hydrolysis for 24h at 50 ºC. The enzymatic reaction was stopped by heating the sample at 90-100 ºC for 10 min followed by immediate cooling in an ice bath. The enzymatic aqueous solutions were then centrifuged, filtered and frozen according to HWE procedures. The pH of all extracts was adjusted to pH 7.0 with 1M HCl and/or NaOH before freezing. The frozen extracts were lyophilised, weighed and stored in desiccators in the dark at room temperature. The extraction yield was calculated as weight percentage of lyophilised extract to the dried seaweed submitted to extraction.

Table 5.1. Summary of optimum hydrolysis conditions, characteristics and enzyme sources.

Enzyme	Optimum conditions[a]		Characteristics	Brand/Source
	pH	Temperature (ºC)		
Alcalase	8.0	50	Endo-peptidase	Sigma-Aldrich *Bacillus licheniformis*
Flavourzyme	7.0	50	Endo-protease and exo-peptidase	Sigma-Aldrich *Aspergillus oryzae*
Cellulase	4.5	50	Cellulase	Sigma-Aldrich *Aspergillus niger*
Viscozyme® L	4.5	50	Multi-enzyme complex of carbohydrases: arabanase, cellulase, β-glucanase, hemicellulase, and xylanase	Sigma-Aldrich *Aspergillus* sp.

[a]Information based on Wang et al. (2010) and Heo et al. (2005); pH of *S. muticum*, *O. pinnatifida* and for *C. tomentosum* water solutions before extraction was 5.6, 4.3 and 6.6, respectively.

5.2.3. Proximate characterization of the extracts

The content in moisture, organic matter, ash, nitrogen and protein, total fat, total sugar as well as total polyphenols in the extracts were determined according to procedures described in chapter 3, section 3.2.2.1. The content of sulphate groups was determined by turbidity through the barium chloride–gelatin method (Dodgson, 1961) using Na_2SO_4 as a standard (0- 200 µg/mL).

5.2.4. Determination of antioxidant activity of the extracts

Total antioxidant capacity. Total antioxidant capacity of extracts' solutions was measured according to method described by Gião et al. (2007). This method is able to quantify both water and lipid-soluble antioxidants, as pure compounds or in crude extracts via direct production of the $ABTS^{\bullet+}$ chromophore (blue/green) by reaction of ABTS and potassium persulphate. To 2 mL of diluted $ABTS^{\bullet+}$ solution it was added 120 μL of extract solution (2 mg lyophilized solids//mL) and absorbance at 734 nm was measured (Asample). Three replicates were performed. Using ascorbic acid as standard (0 to 100 μg/mL), the results were expressed as equivalent concentration of ascorbic acid ($\mu g_{ascorbic\ acid\ equiv}$/mL). The percentage of scavenging activity was also determined using the following formula:

$$\text{Scavenging } \% = \left(\frac{\text{AABTS}^{\bullet+} - \text{Asample}}{\text{AABTS}^{\bullet+}}\right) * 100$$

For each sample the initial absorbance of 2 mL of diluted $ABTS^{\bullet+}$ was measured ($AABTS^{\cdot+}$).

DPPH-free-radical scavenging activity. The DPPH-free-radical scavenging activity was measured according to the method described by Suresh et al. (2013). An aliquot (0.1 mL) of each extract (2mg lyophilized solids/mL) was added to 3.0 mL of 0.1 mM ethanolic DPPH solution and absorbance was measured at 517 nm after incubation for 30 min at 30 ºC in the dark (Asample). Three replicates were performed. Using cathecol as standard (0 to 50 μg/mL), the results were expressed as equivalent concentration of cathechol ($\mu g_{catghecol\ acid\ equiv}$/mL) whereas the percentage of scavenging activity was also calculated using the following formula:

$$Scavenging\ \% = \left(1 - \frac{Asample - Ablank}{Acontrol}\right) * 100$$

For each sample, the absorbance of 3 mL of DPPH plus 0.1 mL ethanol was measured as control (Acontrol), whereas the absorbance of 3 mL of ethanol plus 0.1mL of extract was measured as the blank (Ablank).

Hydroxyl-radical scavenging activity. Hydroxyl-radical ($OH^{\bullet}$) scavenging activity was measured according to the method described by Sudha et al. (2011) based on Smirnoff & Cumbes (1989). An aliquot (1 mL) of each extract (2 mg lyophilized solids /mL) was added to 2 mL of reaction mixture containing 1mL of 1.5 mM FeSO4, 0.7 mL of 6 mM hydrogen peroxide and 0.3 mL of 20 mM of sodium salicylate. After incubation for 1 hour at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm (Asample). Three replicates were performed. Using ascorbic acid as standard (0 to 100 μg/mL), results were expressed as equivalent concentration of

ascorbic acid equivalent ($\mu g_{ascorbic\ acid\ equiv}$/mL) whereas the percentage of scavenging activity of hydroxyl radical was also calculated using the following formula:

$$Scavenging\ \% = (1 - \frac{Asample - Ablank}{Acontrol}) * 100$$

For each sample, the absorbance of 2 mL reaction mixture plus 1 mL of deionized water was measured as the control (Acontrol) whereas 2 mL of reaction mixture, with sodium salicylate substituted by water plus 1 mL of extract was measured as blank (Ablank).

Superoxide-radical scavenging activity. Superoxide-anion-radical ($O_2^{-\bullet}$) scavenging activity was based on the capacity of the extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the riboflavin-light-NBT system according to the method described by Fiorentino et al. (2007). An aliquot (0.1 mL) of each extract (0.2 mg lyophilized solids /mL) was added to 3 mL of reaction mixture containing 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 2μM riboflavin, 100 μM EDTA, 75 μM NBT. After 20 min illumination (fluorescent lamp) the absorbance was measured at 560 nm (Asample). Three replicates were performed. Using gallic acid as standard (0 to 15 μg/mL), results were expressed as equivalent concentration of gallic acid equivalent (μg $g_{gallic\ acid\ equiv}$ /mL) whereas the percentage of scavenging activity of hydroxyl radical was also calculated using the following formula:

$$Scavenging\ \% = (1 - \frac{Asample - Ablank}{Acontrol}) * 100$$

For each sample, the absorbance of 3 mL of reaction mixture plus 0.1 mL deionized water was measured as control (Acontrol), whereas the absorbance of 3 mL of reaction mixture plus 0.1mL of extract without light incidence throughout the 20 min was measured as the blank (Ablank).

5.2.5. Determination of prebiotic potential of seaweed extracts

The evaluation of potential prebiotic activity of seaweed extracts was performed by enumeration of viable cells of two probiotic strains namely *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* BB12 in MRS broth without conventional carbon source and supplemented with seaweed extracts (6%) throughout 48h at 37 ºC. The probiotic cultures of *B. animalis* BB12 and *L. acidophilus* La-5 were obtained as freeze-dried cultures from CHR-Hansen (Denmark). Growth of both probiotic bacteria were also evaluated in MRS broth with glucose (6%), with fructooligosaccharides (FOS) (6%) as well as without glucose. All media were sterilized at 110 °C for 10 min, and after cooling were added with filter-sterilized 0.5 g/L L- cysteine-HCl and were inoculated with a 24-h probiotic culture (2%). Two replicates of inoculated media were incubated at 37 °C under agitation and sampled at 0, 4, 8, 12, 24 and 48h. At each sampling time, decimal

dilutions using sterile 0.1 % (w/v) peptone water were plated with 20 µL aliquots, in duplicate, on MRS agar containing 0.5 g/L L- cysteine-HCl. The viable cells were enumerated according to Miles and Mishra method (1938) after incubation at 37 °C for 48h under under anaerobic conditions with GENbox.

5.2.6. Determination of α-Glucosidase inhibitory activity of the extracts

The α-Glucosidase inhibitory activity was determined in 96 well plates according to the method described by Kwon et al. (2008). The extracts (50µL) at the concentration of 10 mg lyophilised solids/mL, were mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-Glucosidase solution (1.0 U/mL), and pre-incubated at 25ºC for 10 min. Then, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at 5s intervals. The reaction mixtures were incubated at 25ºC for 5 min, and the absorbance readings were recorded at 405 nm by a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany) and compared to a control which had 50 µL of buffer solution in the place of the extract. Acarbose (Sigma) was used as positive control at the concentration of 10 mg/mL. The α-Glucosidase inhibitory activity was expressed as inhibition (%) and was calculated as follows:

$$\%\, Inhibition = \left(\frac{\Delta Abs, control - \Delta Abs, sample}{\Delta Abs, control}\right) * 100$$

Where ∆Abs, control is the variation of absorbance of the control and ∆Abs, sample is the variation of absorbance of the samples.

5.2.7. Statistical analysis

Data are expressed as the mean plus standard deviation of replicates. Two-way ANOVA was performed for each parameter analysed, except for prebiotic activity, with SigmaStat™ (Systat Software, Chicago, IL, USA) to assess if seaweeds species and extraction method were a significant source of variation, at a significance level of p=0.05. Subsequently, and since significant differences and significant interactions were recorded, one-way ANOVA was carried out for each seaweed species to assess whether each extraction method was a significant source of variation for the parameters analysed, at a significance level of p=0.05. The Holm-Sidak method was used for pair-wise comparisons at a significance level of p=0.05.

In terms of prebiotic activity, for each probiotic bacterium, a three-way ANOVA was carried out with SigmaStat™ (Systat Software, Chicago, IL, USA), to assess whether the seaweed species, extract and incubation time at 37 ºC were significant sources of variation at a significance level of p=0.05. Since significant differences were observed for each factor (seaweed species, extract and incubation time) as well as significant interactions, one-way ANOVAs were carried out for each

seaweed species to observe if the carbon source (glucose, FOS or extract) were statistically significant for the number of viable cells of *L. acidophilus* La-5 or *B. animalis* BB12, after 24 or 48h of incubation, respectively. One-way ANOVAS were also performed to evaluate statistical significance of the viable cells after 24 and 48h, in comparison to values at 0h.

5.3. Results and Discussion

5.3.1. Extraction yield

The extraction yields achieved for each of the different aqueous extractions of the three seaweeds are displayed in Figure 5.1. Both seaweed species and extraction method were shown to have a significant effect on extraction yield (p<0.05). In general, in terms of species higher extraction yields were observed for *C. tomentosum* (green seaweed) followed by *O. pinnatifida* (red seaweed) and *S. muticum* (brown seaweed) extracts.

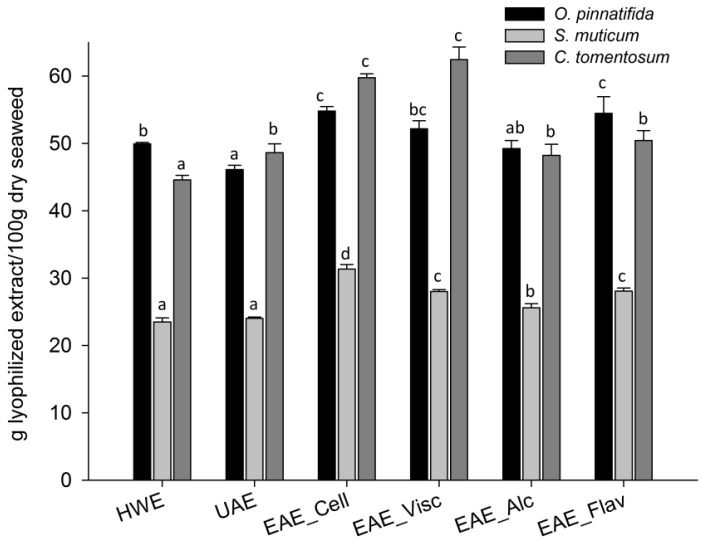

Figure 5.1. Extraction yields of *O. pinnatifida*, *S. muticum* and *C. tomentosum* extracts obtained by hot water extraction (HWE), ultrasound-assisted extraction (UAE) and enzyme-assisted extraction (EAE) with Cellulase (Cell), Viscozyme (Visc), Alcalase (Alc) and Flavourzyme (Flav) at 50 ºC. Different letters indicate significant differences (p<0.05) between extracts for each seaweed.

In general, an inverse correlation between the organic matter of the seaweed and the respective extraction yields was observed; 67, 58 and 55% of organic matter was reported for *S. muticum*, *O. pinnatifida* and for *C. tomentosum*, respectively (Chapter 3, Table 3.1). According to Wijesinghe & Jeon (2012), the amount, diversity and complexity of polysaccharides in the seaweeds' cell wall could reduce the extraction efficiency especially with classical extraction methods. In terms of extraction method EAE extracts were shown to be the most effective. The extraction efficiency of compounds from seaweeds is limited due to the presence of complex cell walls and therefore, the degradation of their structure is a fundamental step for the release of compounds from seaweeds.

Hydrolytic enzymes have been reported to improve the extraction yield in seaweeds (Wang et al., 2010), an effect which was corroborated with the results reported herein for all three seaweed types studied.

In general, higher extraction yields were observed when assisted by both types of enzymes (carbohydrases and proteases) than in hot water-based extracts or ultrasound-assisted extracts, especially in the case of *S. muticum* and *C. tomentosum*. Higher yields were also reported by Hardouin et al. (2014) for *S. muticum* extracts obtained with proteases and carbohydrases than with hot water. To my best of knowledge, there are no studies involving EAE of functional ingredients from *O. pinnatifida* or *C. tomentosum*, which hinders any further comparison besides that achieved within this study, Cellulase and Viscozyme were responsible for the statistically significant highest extraction yields (60-62%) in *C. tomentosum* (Figure 5.1, $p<0.05$) whereas no statistical differences were found in extraction by both proteases (Alcalase and Flavourzyme) or by ultrasound procedures (49-50%, $p>0.05$) in comparison to HWE. Apparently Cellulase and the multi-carbohydrase complex actions of Viscozyme on *C. tomentosum* cell wall polysaccharides were responsible for higher solid content in lyophilized extracts. Extraction yields with Cellulase and Viscozyme were of similar magnitude to those observed by Wang et al. (2010) with red seaweed *Palmaria palmata* which were around 60%. Complex polysaccharides such as alginates, agar, carrageenans, α- and β-D-mannans, are practically non-digested by human gastrointestinal enzymes, whereas, microorganisms' derived-enzymes such as some types of carbohydrases are able to degrade complex polysaccharides converting water-insoluble seaweeds into water-soluble materials collected in water-based extracts (Heo et al., 2003).

Similar tendencies such as those for *C. tomentosum* but with lower extraction yield values were observed for *S. muticum* (Figure 5.1). Cellulase was responsible for the statistically significant highest extraction yield of 31.3% ($p<0.05$). This value is in the range of extract yield (30 to 40%) reported by Plaza et al. (2010) with subcritical water extraction (SWE) at 100 and 200 ºC, and by Hardouin et al. (2014) with EAE (Cellulase) for *S. muticum,* respectively. Although SWE is known for its speed, high yields, and the use of reduced amount of solvents, EAE with Cellulase may also provide several advantages given the enzyme versatility and robustness; furthermore, similar extraction efficiency of targeted compounds and bioactivity properties is achieved using conventional equipment.

No statistically significant differences ($p>0.05$) were found between *S. muticum* extracts obtained from Viscozyme (28.0%) and Flavourzyme (28.1%) and between extracts obtained from UAE (24.0%) and HWE (22.5%) methods. Alcalase action resulted in the lowest extraction yield (25.6 %) within the EAE methods applied.

In *O. pinnatifida,* Cellulase and Flavourzyme were responsible for the statistically significant highest extraction yields (54-55%, $p<0.05$) whereas no statistically significant differences ($p>0.05$) were found between extracts obtained from Alcalase (49.2%), UAE (49.1%) and HWE (49.9%) treatments. The hydrolytic action of the endo-protease and exo-peptidase in the Flavourzyme enzymatic complex from *Aspergillus oryzae* (Table 5.1) on peptide bonds of *O. pinnatifida* proteins

could be responsible, at least in part, for the higher extraction yield given its highest protein content (23.8%) in comparison to *S. muticum* and *C. tomentosum* (16.9-18.8%, respectively) (Chapter 3, Table 3.1). According to Cian et al. (2013) proteolysis promoted by Flavourzyme in *Porphyra columbina* (red seaweed) residual cake, enhanced extraction of peptides.

UAE is considered an emerging potential technology being considered an efficient alternative to traditional extraction techniques that has been successively used in the plant extraction field (Chemat et al., 2011). However, in the present study UAE was not responsible for higher extraction efficiency than HWE for *O. pinnatifida* and *S. muticum*, yet in the case of *C. tomentosum* it enabled a slightly higher, and statistically significant, value (48.6%) than HWE (44.6%). Since UAE was applied to water extracts after 24h at 50 ºC it was expected an improved yield extract resulting from ultrasound waves due to the implosion of cavitation bubbles. In addition, it has been reported that UAE should be carefully used since unstable compounds such as carotenoids could be degraded (Zhao et al., 2006). Lee et al. (2013) evaluated potential use of UAE in water and methanolic extracts of *Ecklonia cava* (brown alga) and reported slightly higher extraction yield values after 6 and 12h at 30 ºC in a ultrasonic bath with 200W (31.3-34.3%) than after 24h in a shaking incubator at room temperature (28.7%).

5.3.2. Proximate characterization of the extracts

In general, and as expected, enzymatic extracts resulted from proteases action presented higher nitrogen content (Table 5.2); Alcalase and Flavourzyme were responsible for higher content in *S. muticum* (25-30 $mg/g_{lyoph\ extract}$; $p<0.05$), *O. pinnatifida* (15-19 $mg/g_{lyoph\ extract}$; $p<0.05$) and in *C. tomentosum* (22-24 $mg/g_{lyoph\ extract}$; $p<0.05$). Indeed, proteases appeared as the most effective enzyme for protein recovery and accessibility.

In terms of sugars, EA extracts obtained from carbohydrases action presented higher content of sugars; Cellulase was responsible for higher content in *S. muticum* (87 $mg_{glucose\ equiv}/g_{lyoph\ extract}$; $p<0.05$) and in *O. pinnatifida* (102 $mg_{glucose\ equiv}/g_{lyoph\ extract}$; $p<0.05$) whereas higher values were obtained by Viscozyme action in *C. tomentosum* (157 $mg_{glucose\ equiv}/g_{lyoph\ extract}$; $p<0.05$).

The different yields of nitrogen and sugar content in the extracts for the 3 species (Table 5.2) did not correlate with the seaweeds proximate composition in terms of total protein and total sugars content. For both parameters, an inverse correlation between their content in the enzymatic extracts and the seaweed composition was observed: statistical significant higher values of protein content was observed for *O. pinnatifida* (23.8 g/100 $g_{dry\ seaweed}$) and the lowest for *S. muticum* (16.9 g/100 $g_{dry\ seaweed}$); in terms of total sugars *S. muticum* was characterized by the highest content in sugar (49.3 g/100 $g_{dry\ seaweed}$) than *O. pinnatifida* and *C. tomentosum* with similar content of 32-33 g/100 $g_{dry\ seaweed}$ (Chapter 3, Table 3.1).

No statistical differences were observed for both nitrogen and sugar contents in the extracts obtained with HWE and UAE for the three seaweeds except for *O. pinnatifida* for which the nitrogen content was higher in the HWE extract than in the UAE counterpart ($p<0.05$). These results may

indicate that the mechanical action resulting from ultrasound waves under the extraction time and temperature conditions used did no promote the release of extra proteins or polysaccharides from the intracellular or wall contents of seaweeds.

Table 5.2. Contents of nitrogen, sugars, sulphated sugars and total phenolics in the different extracts of *S. muticum*, *O. pinnatifida* and *C. tomentosum* seaweeds.

Seaweed	Extraction method	Nitrogen ($mg/g_{lyoph\ extract}$)	Sugars ($mg_{glucose\ equiv}/g_{lyoph\ extract}$)	Sulphated sugars ($mg_{Na2SO4\ equiv}/g_{lyoph\ extract}$)	Total phenolic content ($\mu g_{cathecol\ equiv}//g_{lyoph\ extract}$)
S. muticum	HWE	24.6±3.58^{b}	40.6±2.45^{b}	9.5±0.36^{c}	275.8±4.98^{b}
	UAE	24.4±0.33^{b}	37.8±0.16^{b}	10.1±0.27^{c}	235.0±5.57^{a}
	EAE_Cell	22.1±0.43^{ab}	87.3±4.82^{c}	5.9±0.40^{a}	261.9±29.42^{ab}
	EAE_Visc	25.1±0.62^{b}	29.9±1.95^{a}	7.6±0.61^{b}	300.3±11.67^{b}
	EAE_Alc	29.6±0.01^{bc}	30.7±0.39^{a}	7.5±0.50^{b}	289.7±18.46^{b}
	EAE_Flav	25.4±0.74^{b}	34.7±2.37^{ab}	9.7±0.77^{c}	200.5±7.40^{a}
O. pinnatifida	HWE	12.7±0.50^{b}	73.9±5.15^{b}	41.4±1.30^{b}	118.6±5.98^{a}
	UAE	10.3±0.02^{a}	83.2±6.56^{b}	42.8±1.64^{b}	103.7±1.67^{a}
	EAE_Cell	11.7±0.50^{ab}	102.2±5.34^{c}	41.4±0.81^{b}	112.4±0.27^{a}
	EAE_Visc	12.5±0.53^{b}	71.3±6.48^{b}	59.8±1.20^{d}	109.2±2.17^{a}
	EAE_Alc	15.5±1.04^{c}	59.5±3.06^{ab}	38.6±1.25^{ab}	106.0±2.92^{a}
	EAE_Flav	19.4±0.14^{d}	76.0±5.16^{b}	46.0±1.97^{bc}	123.1±21.89^{a}
C. tomentosum	HWE	17.7±0.19^{b}	62.9±2.38^{b}	11.7±0.20^{c}	107.6±3.13^{a}
	UAE	19.0±0.54^{b}	69.3±5.73^{b}	11.7±0.25^{c}	141.1±9.79^{b}
	EAE_Cell	13.1±0.07^{a}	102.93±5.68^{c}	10.5±0.29^{b}	108.2±5.94^{a}
	EAE_Visc	13.8±1.37^{a}	156.7±4.55^{d}	12.1±0.16^{c}	125.6±8.56^{ab}
	EAE_Alc	22.2±0.36^{c}	40.9±2.17^{a}	9.9±0.25^{b}	121.8±7.64^{ab}
	EAE_Flav	24.1±0.41^{c}	56.8±0.47^{ab}	9.3±0.33^{a}	159.0±11.25^{b}

For each seaweed, different letters indicate significant differences (p<0.05) between methods of extraction

Variable content in sulphate groups, which was significantly different for each seaweed species and extraction method (p<0.05), is observable in Table 5.2. Previous studies with FTIR-ATR analysis showed that in the brown seaweed *S. muticum,* alginates and fucoidans were the main polysaccharides whereas *O. pinnatifida* was mostly an agar producer; sulphate esters of sulphated polysaccharides were observed in the spectrum of *C. tomentosum* (Chapter 3, section 3.3.4). Therefore the sulphated content in each extract was in part related with the nature and amount of sulphated polysaccharide in each seaweed species as well as with the extraction efficiency.

High content of sulphate groups was observed in *O. pinnatifida* extracts (39-60 $\mu g_{Na2SO4\ acid\ equiv}/g_{lyoph\ extract}$) probably from agar, a less sulphated polysaccharide than carrageenans also characteristic in some red seaweeds. A significantly higher content was obtained with EAE using Viscozyme. According to Domozych (2011), red seaweeds possess complex composite cell walls which are made of cellulose, xylan or mannan fibrils as well as an extensive matrix of polysaccharides such as agar and carrageenan.

For *S. muticum* extracts, enzymatic activity did not result in higher content of sulphate groups except for extraction performed with Flavourzyme, in accordance to data reported by Hardouin et

al. (2014) for this seaweed. Hot water and UA extracts revealed to be more suitable to obtain extracts richer in the sulphate groups. These probably result from fucoidans. The presence of sulphated ester groups (S=O), characteristic component in fucoidan and other sulphate polysaccharides, was assigned by FTIR-ATR for *S. muticum* (Chapter 3, section 3.3.4). According to Ale and Meyer (2013) fucoidans cover several different structural fucose-containing sulphated polysaccharides, comprising many different types of fucose-rich polysaccharides, including sulphated fucogalacturonans found in *Sargassum* sp., for example. The lower content of sulphated groups in *S. muticum* in comparison to *O. pinnatifida* could be related with presence of alginates (a non-sulphated polysaccharide), also evidenced by FTIR-ATR analysis. *Sargassum muticum,* an original species from Japan now found in the Atlantic Ocean and in the Mediterranean Sea (invasive seaweed), has been reported to possess, when dry, 10-18% alginates (Guiry, 2014). A slightly higher content in sulphated groups, in comparison to *S. muticum,* was observed in *C. tomentosum* extracts. These could be related to the presence of sulphated xyloarabinogalactans such as sulphated arabinan and sulphated arabinogalactan which have been identified in *C. tomentosum* (Shanmugan & Mody, 2000) or other sulphated polysaccharides (Chapter 3, section 3.3.4). Extracts that resulted from EAE with Cellulase and Viscozyme, as well as from HWE and UAE, were those with higher content in sulphated groups.

Total phenolic content varied significantly according to extraction mode and seaweed type (p<0.05). Higher values for total phenolic content were found for *S. muticum* extracts which ranged between 201 to 301 $\mu g_{\text{cathecol equivalent}}/g_{\text{lyoph extract}}$ (Table 5.2). Once again no direct correlation was observed between total phenolic content, in each seaweed, and those in the respective aqueous extracts. Significant differences in total phenolic content was observed between the three seaweed species namely 460, 250 and 169 $\mu g_{\text{cathecol equivalent}}/g_{\text{dry seaweed}}$ for *C. tomentosum*, *S. muticum* and *O. pinnatifida*, respectively (Chapter 3, Table 3.1). This observation might suggest that none of the extraction modes were sufficiently efficient to extract all phenolics in *C. tomentosum* and *O. pinnatifida.* It has been reported that organic solvents such as ethanol are more efficient for polyphenol extraction than water (Farvin & Jacobsen, 2013).

Variable efficiency of phenolic compounds extraction was observed by the different extraction modes for each seaweed species. For *S. muticum* statistical higher values were obtained by HWE and by Viscozyme and Alcalase EAE (Table 5.2). For *C. tomentosum*, UAE and Flavourzyme EAE were responsible for higher efficiency whereas for *O. pinnatifida* no statistical difference were observed for the different extracts. No information on effects of water-based extraction mode on phenolic compounds was found in the literature. For example higher content in total phenols values was reported by Plaza et al. (2010) in *S. muticum* extracts obtained by SWE water extracts at 100 and 200 ºC ranging between 10.7 and 58.67 mg $_{\text{gallic acid}}/g_{\text{extract}}$, respectively, but no explanation is given for the increment in total phenols except that with a higher extraction temperature a higher mass transfer occurs which increases extraction yield. Water and ethanolic extracts of 16 species collected at the Danish Coast were screened for extraction yield and respective total phenolic content by Farvin & Jacobsen (2013). For all species, water extracts showed higher yield but in

terms of phenolic content this was dependent on the species. In general, the ethanolic extracts had higher content of total phenolics except for 4 species including *S. muticum* (Farvin & Jacobsen, 2013). No explanation is yet available for the higher extractability of phenolic compounds by water in some species such as in *S. muticum*. In turn, for *O. pinnatifida*, methanolic extraction rendered a higher extraction yield and higher polyphenol content than by aqueous extraction (Jiménez-Escrig et al., 2012). According to Budhiyanti et al. (2012) phenolic content varied from 0.006 to 0.65 and from 6.72 to 21.99 $g_{phloroglucinol\ equiv}/100g_{dried\ extract}$ for cytoplasmic and membrane bound extracts from *Sargassum* sp., respectively. These data show that the phenolic content are extremely variable among species of *Sargassum* and cell structure.

5.3.3. Antioxidant activity of the extracts

The figure 5.2 presents data for total antioxidant capacity, DPPH-free-radical as well as for hydroxyl and superoxide-radical scavenging activities of the different extracts for each seaweed, evaluated both as μg of phenolic compound equivalent or % of scavenging activity. Since the diverse expression of results for the different antioxidant capacity evaluation methodologies found in literature makes comparison between studies difficult (Freitas et al., 2013), the authors think that the perception of the relative proportion between phenolic content (μg/mL) of extract and the correspondent value in terms of percentage of scavenging activity turns the interpretation more objective and more valuable (Figure 5.2). The 4 different antioxidant evaluations varied significantly among seaweed species and among the extraction mode (p<0.05). Maximum total antioxidant capacity as well as DPPH-free-radical and hydroxyl-radical scavenging activities were observed in the extracts of *S. muticum* followed by *C. tomentosum* (Figure 5.2). In *O. pinnatifida* extracts, lower total antioxidant capacity and DPPH-free-radical scavenging activity were observed in comparison to any of the two other seaweeds but higher scavenging capacity for reactive oxygen species such as $HO^{\bullet}$ and in particular of radical $O_2^{-\bullet}$ were achieved. Heo et al. (2005) also reported variable scavenging activities in brown seaweed extracts obtained by carbohydrases or proteases in terms of DPPH free, hydroxyl and superoxide radicals. According to Freitas et al. (2013) no single assay seems to be able to accurately reflect the mechanism of action of all radicals and antioxidants especially in complex systems. For example some antioxidants may not be efficient quenchers of peroxyl radicals but only be singlet-oxygen scavengers (Karadag et al., 2009).

The statistical significant difference between the seaweeds extracts (p<0.05), could be, at least in part, attributed to the higher amount of total phenolic compounds and of proteinaceous compounds found in *S. muticum* (Table 5.2). The radical scavenging activity of phenolic compounds is however dependent on the phenolic structure and number and location of hydroxyl groups (Balboa et al., 2013) and therefore the radical scavenging potential of the extracts is dependent of qualitative and quantitative phenolic combination. These results are in accordance to those published by Yangthong et al. (2009) which reported higher phenolic content and antioxidant activity for brown seaweeds than for red and green seaweeds.

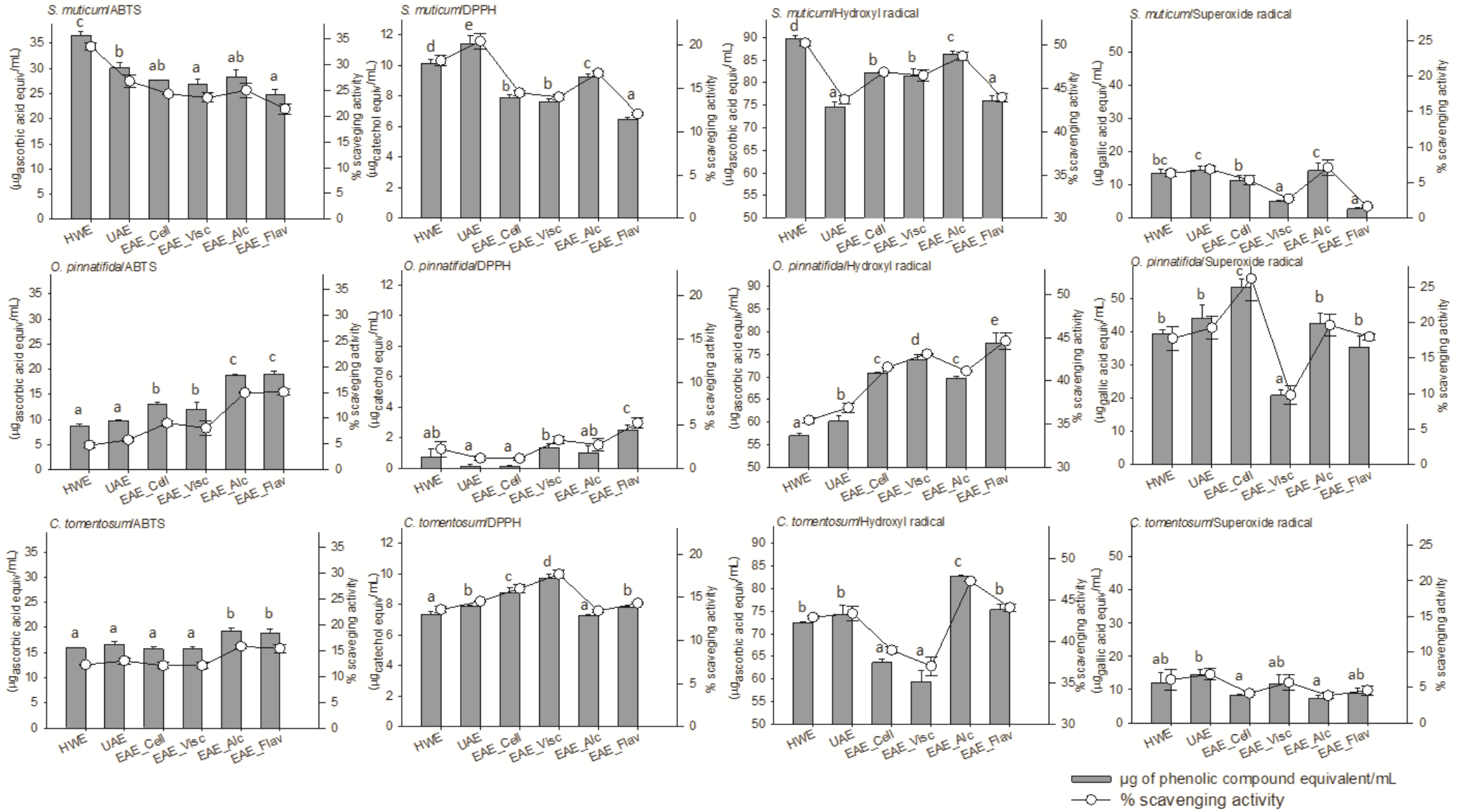

Figure 5.2. Total antioxidant capacity, DPPH free radical, Hydroxyl radical and superoxide radical scavenging activities of *S. muticum*, *O. pinnatifida* and *C. tomentosum* extracts obtained by hot water extraction (HWE), ultrasound-assisted extraction (UAE) and enzyme-assisted extraction (EAE) with Cellulase (Cell), Viscozyme (Visc), Alcalase (Alc) and Flavourzyme (Flav) at 50 ºC. Different letters indicate significant differences ($p<0.05$) between extracts for each seaweed.

Polysaccharides in brown seaweed such as those with high content of uronic acid and alginates with low molecular weight have also been reported as good antioxidant potential (Zhao et al., 2012).

Antioxidant potential of *S. muticum* water extracts was higher in HWE especially in terms of total antioxidant capacity (p<0.05) with 33.5% scavenging activity (corresponding to 37 $\mu g_{ascorbic\ acid\ equiv}$/mL) and hydroxyl-radical scavenging activity (p<0.05) with 50.3% scavenging activity (corresponding to 90 $\mu g_{ascorbic\ acid\ equiv}$/mL), respectively. Maximum DPPH-free-radical scavenging activity was observed for UAE with 20% scavenging activity (corresponding to 11 $\mu g_{cathecol\ equiv}$/mL). In terms of antioxidant activity no increase was observed in the *S. muticum* extracts obtained from EAE. Higher values of free and hydroxyl scavenging activity was reported by Je et al. (2009) in EA extracts with Alcalase and Viscozyme from brown seaweed *Undaria pinnatifida* but no hydroxyl scavenging activity from EA extracts with Flavourzyme was reported. Hydroxyl scavenging activity of *S. muticum* extracts produced with Flavourzyme was of 42%, one of the lowest values together with those obtained for UAE extracts (Figure 5.2). In *O. pinnatifida*, EA extracts had, in general, higher scavenging potential than HWE or UAE. In terms of total antioxidant capacity, proteases assisted extracts with 19% scavenging activity (corresponding to 15-16 $\mu g_{ascorbic\ acid\ equiv}$/mL), presented statistically significant higher values (p<0.05) than the other extraction methods. According to Cian et al. (2013) the low molecular weight peptides and polyphenols released during proteolysis were responsible for the DPPH radical scavenging activity of red seaweed *Porphyra columbina*. No radical scavenging activity towards ABTS was reported by Jiménez-Escrig et al. (2012) for aqueous extracts of *O. pinnatifida*. For the hydroxyl-radical, EA extracts were capable of 42 to 45% scavenging activity (corresponding to 70-79 $\mu g_{ascorbic\ acid\ equiv}$/mL) (Figure 5.2). *O. pinnatifida* EA extracts from Cellulase had a statistically significant (p<0.05) higher superoxide scavenger potential with 28% scavenging activity (corresponding to 54 $\mu g_{gallic\ acid\ equiv}$/mL), than the other extracts.

The extraction procedures did not affect the total antioxidant capacity displayed by *C. tomentosum* extracts except for those obtained by proteases EAE where results were higher and statistically different (Figure 5.2). In terms of superoxide-radical scavenging activity only slight differences were observed between the different *C. tomentosum* extracts. However, Cellulase and Viscozyme extracts of *C. tomentosum,* which had higher sugar content, had statistically significant (p<0.05) higher DPPH-free-radical scavenger activity with 16 to 18% (corresponding to 9-10 $\mu g_{cathecol\ acid\ equiv}$/mL). Valentão et al. (2010) also reported a weak concentration-dependent antioxidant capacity for *C. tomentosum* aqueous lyophilized extracts (10% of DPPH-free-radical scavenging activity with 794 μg/mL extract). In terms of the hydroxyl-radical, Alcalase and Flavourzyme extracts were able to inhibit more than 44% of scavenging activity corresponding to 76 to 83 $g_{ascorbic\ acid\ equiv}$/mL which may indicate that such bioactivity is related not only with total phenolic content but also with proteinaceous compounds (Table 5.2). Nevertheless, given the statistically significant difference between Alcalase and Flavourzyme scavenging capacities and

their respective phenolic and nitrogen contents shown in Table 5.2 it seems that not only the content but also the nature of the compounds are important to eventually justify the antioxidant properties. For example, Heo et al. (2005) reported that enzymatic extracts of brown seaweed *Ecklonia cava* with Alcalase did not possess DPPH-free-radical scavenging activity although they contained as much phenolic compounds as other extracts with free-radical scavenging activity. In turn, Farvin & Jacobsen (2013) reported some extracts with good antioxidant effects but with low total phenolic content indicating that other compounds were co-extracted in water extracts such as sulphate polysaccharides, proteins or peptides. However, not much antioxidant potential was observed in crude sulphated polysaccharides from *Sargassum plagiophyllum* (2mg/mL) for free-radical (11.2%), hydroxyl (11.2%) or superoxide (0%) radical scavenging activities (Suresh et al., 2013).

In summary, the different extracts, with 2 mg lyophilized extract/mL, from *S. muticum*, *O. pinnatifida* and *C. tomentosum* did not exhibit high total antioxidant capacity or DPPH-free-radical scavenging activity but more prominent effects on hydroxyl-radical scavenging activity (35 to 50%). Extracts from *O. pinnatifida* with 0.2 mg lyophilized extract/mL, namely HWE, UAE and EA with Cellulase and Alcalase exhibited moderate effect on superoxide-radical scavenging activity (18-26%).

5.3.4. Prebiotic potential of seaweeds extracts

Recently some research has been done in identifying potential prebiotic compounds in marine resources namely in seaweeds given their rich profile in polysaccharides present in the cell walls (Zaporozhets et al., 2014), nonetheless, to my best of knowledge, no information is available regarding the prebiotic potential of the three seaweed species targeted in this study. Hence, the potential prebiotic effect of seaweed water-based extracts was evaluated by using pure cultures of representative beneficial bacteria including strains of lactobacilli and bifidobacteria (usual target genera for prebiotics) in parallel with well-established prebiotics, such as FOS, for comparison purposes, both probiotic strains were also grown under the same conditions but in the absence of sugar (negative control) as well as in the presence of glucose or FOS at similar concentrations (positive controls) (Figures 5.3 to 5.5). Seaweed species, extraction mode and incubation time were revealed to be significant factors for both probiotic strains viability (p<0.05). Since significant differences were observed for each factor as well as significant interactions, one-way ANOVAs were carried out for each seaweed species to observe if the carbon source (glucose, FOS or extract) was statistically significant for the viable cell numbers of *L. acidophilus* La-5 or *B. animalis* BB12, after 24 or 48h of incubation, respectively. Regarding *L. acidophilus* La-5 significantly higher values (p<0.05) of viable cell numbers were observed for the majority of culture media enriched with seaweed water-based extracts after 24h of incubation in comparison to growth in media with glucose or FOS.

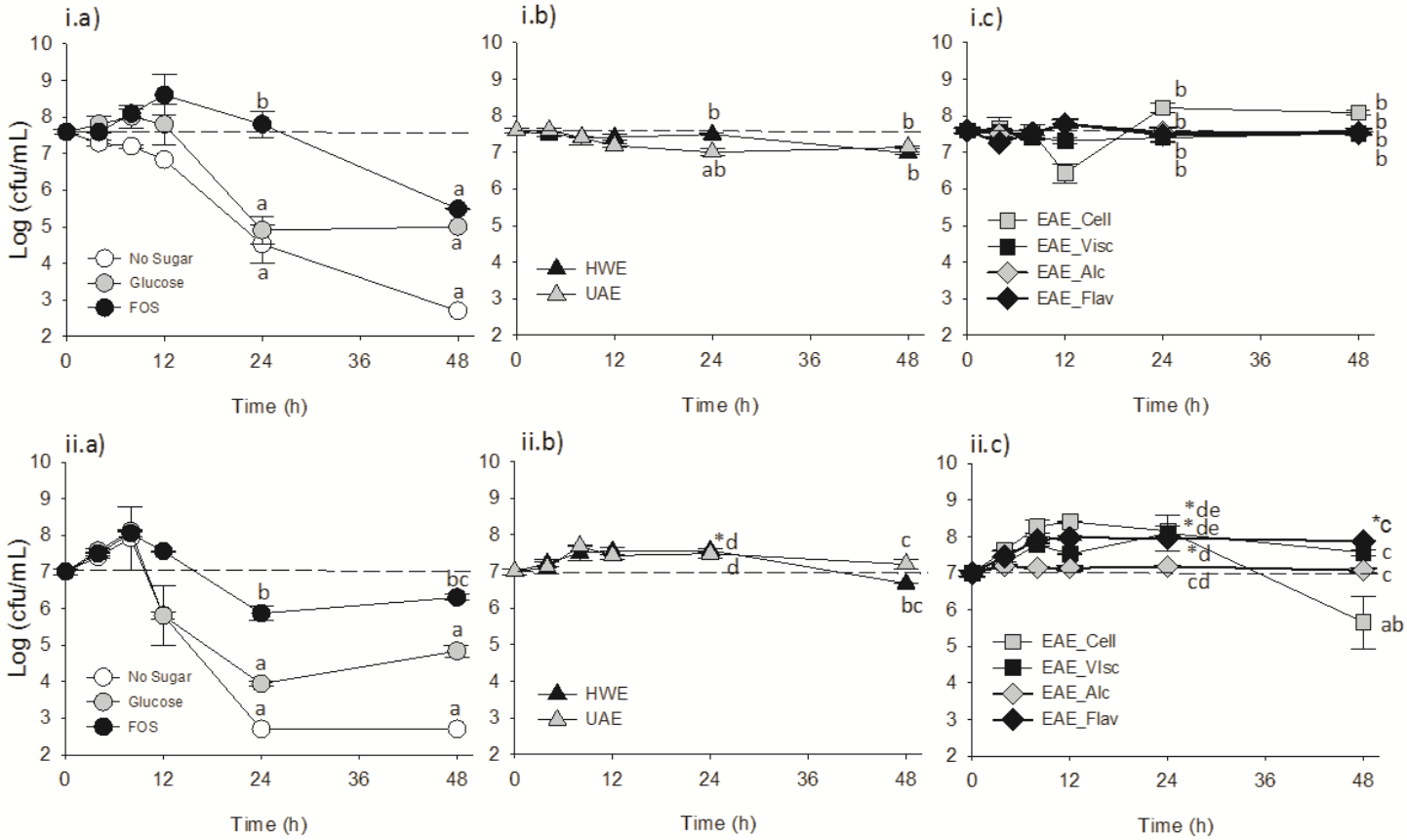

Figure 5.3. Mean and standard deviation of cell counts of *B. animalis* BB12 (i) and *L. acidophilus* La-5 (ii) throughout 48h of incubation at 37 ºC in the presence or absence of sugars (a) or *S. muticum* extracts: hot water extraction (HWE) and ultrasound-assisted extraction (UAE) (b); enzyme-assisted extraction (EAE) with Cellulase (Cell), Viscozyme (Visc), Alcalase (Alc) and Flavourzyme (Flav) (c). Different letters indicate significant differences (p<0.05) for the viable cells after 24 or 48h of incubation at 37 ºC in the presence or absence of sugars or *S. muticum* extracts and * for significant differences (p<0.05) in comparison to values at 0h.

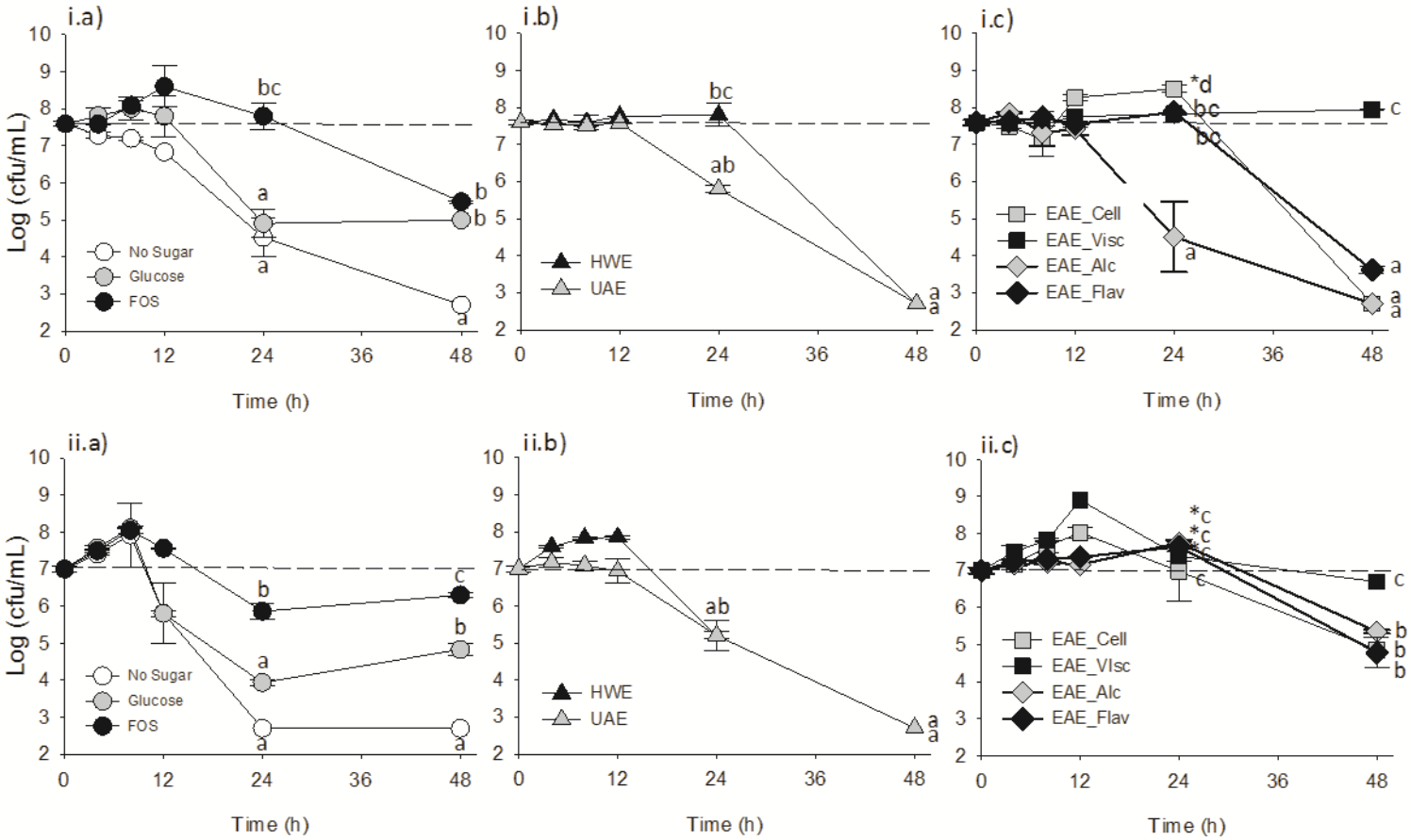

Figure 5.4. Mean and standard deviation of cell counts of *B. animalis* BB12 (i) and *L. acidophilus* La-5 (ii) throughout 48h of incubation at 37 ºC in the presence or absence of sugars (a) or *O. pinnatifida* extracts: hot water extraction (HWE) and ultrasound-assisted extraction (UAE) (b); enzyme-assisted extraction (EAE) with Cellulase (Cell), Viscozyme (Visc), Alcalase (Alc) and Flavourzyme (Flav) (c). Different letters indicate significant differences (p<0.05) for the viable cells after 24 or 48h of incubation at 37 ºC in the presence or absence of sugars or *O. pinnatifida* extracts and * for significant differences (p<0.05) in comparison to values at 0h**.**

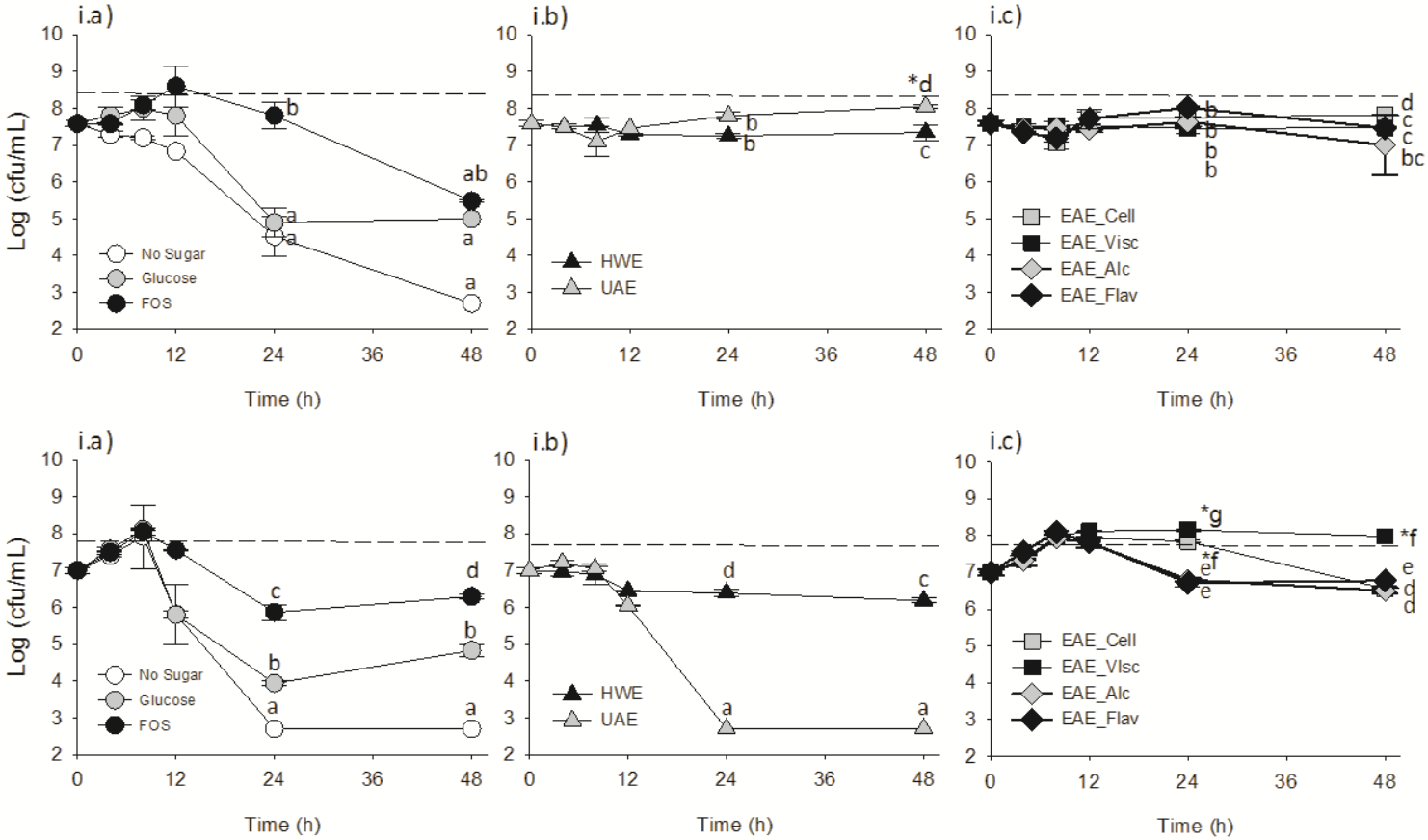

Figure 5.5. Mean and standard deviation of cell counts of *B. animalis* BB12 (i) and *L. acidophilus* La-5 (ii) throughout 48h of incubation at 37 ºC in the presence or absence of sugars (a) or *C. tomentosum* extracts: hot water extraction (HWE) and ultrasound-assisted extraction (UAE) (b); enzyme-assisted extraction (EAE) with Cellulase (Cell), Viscozyme (Visc), Alcalase (Alc) and Flavourzyme (Flav) (c). Different letters indicate significant differences (p<0.05) for the viable cells after 24 or 48h of incubation at 37 ºC in the presence or absence of sugars or *C. tomentosum* extracts and * for significant differences (p<0.05) in comparison to values at 0h.

In addition, some significant increases (p<0.05) in viable cell numbers in comparison to those at 0h were observed such as in media containing HWE and EAE with Viscozyme, Cellulase and Flavorzyme extracts of *S. muticum*, EAE with Viscozyme and Alcalase extracts of *O. pinnatifida* as well as EAE with Viscozyme and Cellulase extracts of *C. tomentosum* (Figures 5.3ii.a-c, 5.4ii.a-c, 5.5ii.a-c).Despite the enhanced growth by 24h incubation, after 48h of incubation lower viable cell numbers were observed, in particular, for *O. pinnatifida* extracts (Figures 5.4ii.b-c); end-product formation or substrate depletion could justify this decrease in viable cell numbers.

In terms of seaweed extracts impact on *B. animalis* BB12, it was found that, in general, they have a lower prebiotic potential (Figures 5.3i.a-c, 5.4i.a-c, 5.5i.a-c). Nonetheless viable cell numbers of *B. animalis* BB12 in all *S. muticum* and *C. tomentosum* extracts after 48h of incubation were significantly higher than those obtained with prebiotic FOS and glucose (p<0.05) (Figures 5.3i.a-c, 5.5i.a-c).

According to the results, the majority of the extracts obtained from the three seaweeds possess carbon sources that can be metabolized by *L. acidophilus* La-5 and *B. animalis* BB12. A more promising prebiotic potential was observed for *S. muticum* and *C. tomentosum* extracts especially for those obtained by enzymatic action. Since a lower potential was observed in extracts of *O. pinnatifida*, this could be related with the largest amount of sulphated sugars observed in these extracts. Red seaweed *O. pinnatifida* has been mostly characterized as an agar producer and it is known that microorganisms are not able to hydrolyse and metabolize this polysaccharide which is used as solidifying agent of culture media. On the other hand, it should not be totally overlooked since it was shown recently that some low molecular weight polysaccharide fractions derived from seaweed agar and alginate by acid hydrolysis or degradation with hydrogen peroxide, were fermented by gut microbiota, namely bifidobacterial populations, exhibiting potential to be used as a novel source of prebiotics (Ramnani et al., 2012).

Enzymatic extracts, especially those obtained with carbohydrases (Viscozyme and Cellulase) probably possess polysaccharides and oligosaccharides derived from hydrocolloids present in each type of seaweed that can act as sources of soluble fibres exerting a prebiotic effect. According to Wu et al. (2012) *Bifidobacterium longum* BCRC 11847 could utilize polysaccharide extracts of red seaweed *Gracilaria* sp. extracted with Cellulase R-10 and Macerozyme R-10 and display an increase in pH-lowering ability. Zaporozhets et al. (2014) recently reviewed the prebiotic potential of polysaccharides and extracts from seaweeds. In this review the authors included their research group studies' results on the prebiotic potential of fucoidan and alginic acid derived from brown seaweed *Fucus evanescens in vitro* and *in vivo* with mice. These authors confirmed that fucoidan and alginic acid were used instead of lactose with a 3 to 5.8-fold increase in biomass of *Bifidobacterium* in comparison to the control.

Given the promising reported results obtained in terms of growth promotion of *L. acidophilus* La-5 and *B. animalis* BB12 and considering the prebiotic potential of polysaccharides and seaweed extracts reported in literature concerning other seaweed species, further *in vitro* (non-digestibility and selective fermentation capacity) must be performed with the most promising extracts.

5.3.5. α-Glucosidase inhibitory activity of the extracts

The α-glucosidase inhibitory activity, expressed as % of inhibition, by *S. muticum*, *O. pinnatifida* and *C. tomentosum* water-based extracts is displayed in Figure 5.6.

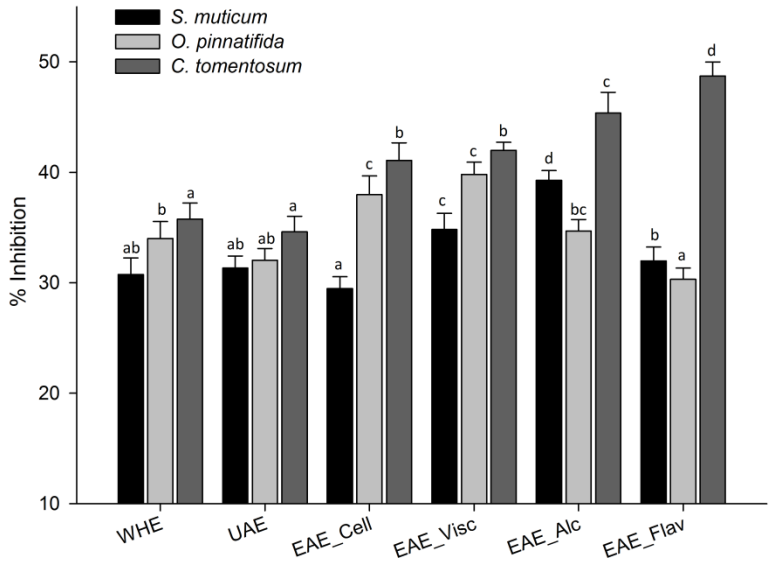

Figure 5.6. α-glucosidase inhibitory activity, expressed as % of inhibition, of *S. muticum*, *O. pinnatifida* and *C. tomentosum* extracts obtained by hot water extraction (HWE), ultrasound-assisted extraction (UAE) and enzyme-assisted extraction (EAE) with Cellulase (Cell), Viscozyme (Visc), Alcalase (Alc) and Flavourzyme (Flav) at 50 ºC. Different letters indicate significant differences (p<0.05) between extracts for each seaweed.

Extraction mode and seaweed species were both statistically significant factors (p<0.05). Extracts obtained by enzymatic action possess higher inhibition activity especially for *O. pinnatifida* with 38 to 40% inhibition in the extracts obtained by Cellulase and Viscozyme and for *C. tomentosum* with 45 to 49% inhibition in the extracts obtained by Alcalase and Flavourzyme. To my knowledge there are no reports on potential anti-diabetic activity of water-based enzymatic seaweed extracts, in particular for the studied species, and therefore no direct comparisons with other works are possible only with other seaweed species and other extraction modes. For example, Senthil et al. (2013) reported that ethyl acetate extracts of red seaweeds of *Chondrococcus harnmanni and P. Gymnospora* (81 and 94%, respectively) possesses high levels of α-amylase inhibitory activity. Lower values of α-glucosidase inhibitory activity were observed for *S. muticum* extracts with a maximum of inhibition in the extracts obtained with Alcalase (39%). This is an interesting observation because it is the *S. muticum* extract with the highest protein content among all other extracts (Table 5.2). Other research studies have studied the role of seaweed polysaccharides namely from other *Sargassum* species, or of phenolic compounds on inhibition activity of α-amylase and α-glucosidase but less on the role of protein or peptides for the same

purpose. For example Hardoko et al. (2014) reported inhibitory activity on α-glucosidase by laminaran and fucoidan fractions from two brown seaweeds (*Sargassum duplicatum* and *Turbinaria decurens*) but not by alginate fraction. According to Motshakeri et al. (2014), 35 to 36% blood glucose reduction was observed in type 2 diabetic rats after 22 days of feeding with 300 mg/kg body weight of ethanolic or water extracts of *Sargassum polycystum*. In the present study, EA extracts from *O. pinnatifida* and *C. tomentosum* showed promising inhibitory potential against α-glucosidase and, although further *in vivo* studies are required to test for effective hypoglycaemic activity, these may prove to be an important strategy in the prevention (functional food) or management (oral therapeutic agent) of blood glucose level in type 2 diabetic and borderline patients.

5.4. Conclusions

In conclusion, the results in this chapter demonstrates once more that edible seaweeds such as *S. muticum* (brown alga), *O. pinnatifida* (red alga) and *C. tomentosum* (green alga) are a natural source of compounds with important biological interest. In the present study, water-based crude extracts were obtained from efficient methods that are also food grade compatible and sustainable. Enzymatic assisted extracts revealed to be promising tool to obtain extracts with attractive biological properties. According to the general results achieved it becomes difficult to pinpoint the most efficient extraction method with the highest biological potential since it depends on several factors: i) seaweed species; ii) extraction mode considering the duality: extraction yield vs chemical/biological properties; and iii) the type of analysis made. In this perspective, *S. muticum* EA presented interesting antioxidant and prebiotic potential, *O. pinnatifida* EA free-radical scavenging activity given its high sulphated sugar content and *C. tomentosum* EA a promising α-glucosidase inhibitory activity and consequently attractive anti-diabetic potential. In general, it can be pointed that the enzyme-assisted extraction seems to be one of the best options in terms of food compatible extraction method with higher yield rates and through the selection of a specific enzyme some biological properties may be favoured over others properties. In terms of functional food development, the selection of the most efficient extraction mode must be coupled to the best results in terms of biological properties according to the intended purpose associated.

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Chapter 6

Bioactive polysaccharides extracts from *Sargassum muticum* by high hydrostatic pressure

6.1. Introduction

Sargassum muticum (Yendo) Fensholt is a brown edible seaweed that can be found along the European waters and on the West Coast of North America (Milledge et al., 2015). As invasive seaweed of difficult eradication it causes negative impacts on ecology, fishing, and recreational activities (Milledge et al., 2015; Balboa et al., 2013). Despite these less positive aspects, the fact that *S. muticum* is available in large quantities (high biomass) and in itself holds important constituents of good nutritional, or even bioactive potential, has led to the development of strategies to valorise such seaweed compounds. Previous studies revealed that *S. muticum* extracts possess various potential biological proprieties, including anti-proliferative, anti-angiogenesis, antimicrobial and anti-inflammatory activities (Namvar et al., 2013; Yoon et al., 2010; Kim et al., 2007) as well as antioxidant and prebiotic (Chapter 5, sections 5.3.3 and 5.3.4). Brown seaweeds are rich in sulphated polysaccharides namely fucoidans and laminarans which have several health benefits associated such as antioxidant activity (Wijesekara et al., 2011).

In general, these compounds may be extracted and/or concentrated and used in different food or nutraceutical applications. Many extraction techniques are available for such purpose yet are not always of favourable application due to associated environmental pollution and costs. Hence, improved extraction techniques are continuously being sought in terms of shortening of operating times, reduction of organic solvent consumption and increase in extraction efficiency. High hydrostatic pressure (HHP) processing, an extraction technique discussed in chapter two, has been considered an emerging non-thermal food processing technique that has shown great promise in food and pharmaceutical industries as well as in biotechnological research (Huang et al., 2013). Therefore this type of extraction was also selected for studding their impact on *S. muticum* extracts characteristics namely in terms of polysaccharides content as an alternative way to the conventional extraction methods. In this chapter six, the main objectives were to assess the potential and effectiveness of HHP to obtain extracts concentrated in bioactive polysaccharides from the brown seaweed *S. muticum* and to optimize extraction parameters, namely, extraction time, pressure, and seaweed solid/liquid ratio using a three level Box-Behnken design. Moreover, to my best of knowledge this is the first study applying HHP to extract bioactive polysaccharides from *S. muticum*. Consequently, an additional objective was to test some biological properties namely total antioxidant capacity, hydroxyl-radical scavenging activity and antidiabetic activity of *S. muticum* polysaccharides extracts obtained via HHP extraction.

6.2. Material and methods

6.2.1. High hydrostatic pressure

Specimens of the brown seaweed (Heterokontophyta, Phaeophyceae) *Sargassum muticum* (Fucales) Sargassaceae family harvested in April 2012 from Buarcos bay (Figueira da Foz,

Portugal) were first washed with running tap water and then with deionized water and then dried in an oven at 60 ºC. The dried seaweeds were milled to less than 1.0 mm particle size and stored in a dark desiccator until further use.

Prior to high hydrostatic pressure (HHP) assisted extraction, dried and milled seaweed was weighed and dispersed in 50 mL of deionized water and placed in an agitating water bath at room temperature for 24h. Subsequently, samples were transferred to polyethylene bags which were heat sealed under vacuum (Albipack Packaging Solutions, Portugal). The bags containing the water extracts were subject to HHP under different time periods and pressures according to the established experimental design (Table 6.1). The HHP-assisted extraction was performed in a hydrostatic press (Hyperbaric 55, Hyperbaric, Burgos, Spain), which has a pressure vessel of 200 mm inner diameter and 2000 mm length with a maximum operation pressure of 600 MPa. It is connected to a refrigeration unit (RMA KH 40 LT, Ferroli, Spain) that enables the control of the input water temperature used as a pressurizing fluid at room temperature (20-22 ºC).

After the HHP processing step, the water extracts were removed from the bags, centrifuged at 5000 g for 10 min at 4 ºC (centrifuge Medifriger BL-S, JP Selecta, Spain), the obtained supernatants were filtered through a glass filter funnel (porosity 1) and the resulting extracts frozen at -80 ºC until freeze-drying.

6.2.2. Design of experiments

The design was constructed and analysed using Minitab® 17 (Minitab® Statistical Software, Pennsylvania, USA) and the response surface plots were plotted by SigmaPlot™ 13 Trial version (USA/Canada).

As shown in Table 6.1, a Box–Behnken design with three independent variables at three levels, namely extraction time (X_1: 5, 17.5 and 30 minutes), extraction pressure (X_2: 300, 450 and 600 MPa), and seaweed solid/liquid ratio (X3: 1, 3 and 5 g of dry seaweed in 50 mL of deionized water) was used for optimization (Box & Behnken, 1960). The range of values for the three independent variables, presented in Table 6.1, were based on the results of preliminary experiments (data not shown); moreover, in the case of extraction pressure the full possible range enabled by the equipment was used in order to enhance the maximum rupture of seaweeds cell walls. Extraction yield, total sugars, total sulphated sugars, total antioxidant capacity and hydroxyl-radical scavenging activity were selected as the response for the combination of the independent variables. The response surface design consisted in 17 runs in randomized order, to minimize the effects of unexpected variability in the observed responses, with five replicates in the centre point to estimate the pure error sum of squares (Table 6.1). The three variables were designated as X_1, X_2, X_3 and prescribed into three levels, coded +1, 0, -1 for high, intermediate and low values, respectively. A full quadratic model was used to fit the data according to Equation (1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+1}^{3} \beta_{ij} X_i X_j \qquad (1)$$

where, Y is the predicted response, β_0, β_i, β_{ii}, and β_{ij} are coefficients in the intercept, linear, quadratic and interaction terms, respectively. X_i and X_j are the independent variables.

Analysis of variance for response surface quadratic model validation was performed, and the test for significance of each term to test for goodness of fit was conducted at $p<0.05$.

6.2.3. Yield and determination of total and sulphated sugars content

The extraction yield was calculated as weight percentage of lyophilized extract (M) to the dried seaweed submitted to extraction (W) as given in Equation (2):

$$Extraction\ yield\ (\%) = \frac{M}{W} \times 100 \quad (2)$$

Total sugars content was determined by the phenol-H_2SO_4 method (Dubois et al., 1956) as described in chapter 3, section 3.2.2.1. The content of sulphate groups was determined by turbidity through the barium chloride–gelatin method (Dodgson, 1961) using Na_2SO_4 as a standard (0- 200 µg/mL) as described in chapter 5, section 5.2.3.

6.2.4. Total antioxidant capacity

Total antioxidant capacity of *S. muticum* extracts was determined according to procedures described in chapter 5, section 5.2.4.

6.2.5. Hydroxyl-radical scavenging activity

Hydroxyl-radical ($OH^{\bullet}$) scavenging activity of *S. muticum* extracts was determined according to procedures described in chapter 5, section 5.2.4.

6.2.6. α-Glucosidase inhibitory activity

The α-Glucosidase inhibitory activity of *S. muticum* extracts was determined according to procedures described in chapter 5, section 5.3.5.

6.3. Results and Discussion

6.3.1. Overview of the efficiency of HHP-assisted extraction of *S. muticum*

Table 6.1 shows the extraction yield, total sugar and total sulphated sugar contents, and antioxidant activity (total antioxidant capacity and hydroxyl-radical scavenging activity) of *S. muticum* extracts prepared under HHP extraction according to Box-Behnken experimental design. Yield values of the extracts ranged between 32.0 and 40.4 g/100g dry seaweed independently of the extraction conditions or seaweed solid/liquid ratio used in the extraction. These values represent a higher yield (i.e. an increment between 36 and 72%) than the *S. muticum* extracts obtained by hot water assisted-extraction (HWE) or by ultrasound-assisted extraction (UAE) (23.5-24.0%; Chapter 5, section 5.3.2). These results demonstrate that high hydrostatic pressure improved overall extraction efficiency from *S. muticum*. According to Briones-Labarca et al. (2015) higher yields were obtained with HHP-assisted extraction from Chilean papaya than UAE and conventional extractions (CE) with organic solvents. Specifically, *S. muticum* extracts from HHP treatment showed a significantly higher level of total sugar and sulphated sugar contents; 142 to 173 $mg/g_{lyophilized\ extract}$ of total sugars were obtained representing a 3.8- to 4.6-fold in comparison to HWE or to UAE of *S. muticum* (37.8-41 $mg/g_{lyophilized\ extract}$; Chapter 5, section 5.3.2). In terms of sulphated sugars a similar trend was observed; 34 to 46 $mg/g_{lyophilized\ extract}$ were obtained representing an increment of 3.6- to 4.8-fold in comparison to HWE or to UAE of *S. muticum* (9.5-10.1 $mg/g_{lyophilized\ extract}$; Chapter 5, section 5.3.2). Sulphated polysaccharides in brown seaweeds are mainly fucans comprising families of polydisperse molecules based on sulphated *L*-fucose, whereas heterofucans are designated fucoidans (Chapter 4, section 3.3.4).

Table 6.1. Box-Behnken matrix along with experimental and predicted values of the responses for the HHP extraction of *S. muticum*

Run No.	Coded levels			Responses								
	X_1	X_2	X_3	Yield		Sugars		Sulphated sugars		$ABTS^{\bullet+}$		$OH^{\bullet}$
	Time (min)	Pressure (MPa)	Seaweed conc. (g/50 mL)	$g_{lyoph\ extract}/100g_{dry\ seaweed}$		$mg_{glucose\ equiv}/g_{lyoph\ extract}$		$mg_{Na2SO4\ equiv}/g_{lyoph\ extract}$		% Scavenging		% Scavenging
				Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	0 (17.5)	+1 (600)	-1 (1)	39.2	39.1	171±7	171.1	39.7±0.7	39.8	30.2±0.5	30.1	34.8±0.3	34.3
2	0 (17.5)	0 (450)	0 (3)	36.3	36.3	155±1	155.4	43±3	42.9	28.9±0.3	29.1	38.1±0.1	38.0
3	+1 (30)	+1 (600)	0 (3)	35.7	35.8	170±1	170.5	41.9±0.4	41.7	27±1	26.7	38.0±0.6	38.9
4	+1 (30)	0 (450)	+1 (5)	32.4	32.5	170±5	169.9	37.3±0.2	37.4	26.7±0.7	26.8	38.0±0.3	37.8
5	0 (17.5)	+1 (600)	+1 (5)	33.8	33.6	159±7	158.8	42.2±0.5	42.4	26.2±0.6	26.3	37.9±0.3	37.2
6	0 (17.5)	0 (450)	0 (3)	36.2	36.3	155±1	155.4	43±1	42.9	29.3±0.6	29.1	37.1±0.1	37.9
7	-1 (5)	0 (450)	-1 (1)	40.2	40.1	160±3	159.6	43±1	43.3	33±1	33.1	31.4±0.3	31.6
8	0 (17.5)	0 (450)	0 (3)	36.6	36.3	155±3	155.4	43±3	42.9	29±1	29.1	38.0±0.1	38.0
9	0 (17.5)	-1 (300)	-1 (1)	40.4	40.6	142±6	141.8	46±3	45.6	33.0±0.8	33.0	32.3±0.6	33.0
10	-1 (5)	-1 (300)	0 (3)	36.5	36.4	148±1	147.8	46±2	45.9	30±2	30.4	34.9±0.8	34.0
11	0 (17.5)	0 (450)	0 (3)	36.6	36.3	155±3	155.4	43±1	42.9	29±5	29.1	38.2±0.9	38.0
12	+1 (30)	0 (450)	-1 (1)	38.7	38.6	163±8	162.8	41.9±0.7	42.1	30.9±0.2	31.2	36.0±0.7	35.6
13	+1 (30)	-1 (300)	0 (3)	36.5	36.4	166±2	166.2	34±2	34.0	28±1	27.5	35.7±0.8	35.4
14	-1 (5)	0 (450)	+1 (5)	31.9	32.0	157±6	157.0	43±4	43.1	28.5±0.8	28.2	34±1	34.9
15	0 (17.5)	0 (450)	0 (3)	36.0	36.3	156±1	155.4	43±2	42.9	28.9±0.9	29.1	38.3±0.2	38.0
16	0 (17.5)	-1 (300)	+1 (5)	32.0	32.0	159±3	158.6	38.1±0.1	38.0	27.5±0.9	27.6	35±1	35.6
17	-1 (5)	+1 (600)	0 (3)	36.8	36.9	173±2	172.8	36.8±0.5	36.8	26.9±0.6	27.1	33.2±0.5	33.5

Responses values for total sugars, sulfated sugars, ABTS and $OH^{\bullet}$ are expressed as mean ± standard deviation

To my knowledge this is the first study applying HHP in preparation of *S. muticum* extracts and there are no published reports regarding the application of this technology to seaweeds so any comparative discussion can only be done in relation to other studies that also employed natural sources including medicinal plants, fruits or mushrooms. For example, Prasad et al. (2010) evaluated the effects of ultra-high-pressure-assisted extraction on longan fruit pericarp at 200, 300, 400 and 500 MPa; highest extraction yields with high phenolic contents and highest antioxidant activities were achieved with 500 MPa In turn, Vega-Gálvez et al. (2014) evaluated the effects of HHP at 300, 400 and 500 MPa through 1, 3 and 5 min on nutritional and antioxidant properties of cape gooseberry pulp after the HHP treatment and after 60 days of storage. Maximum values of total phenolic content and antioxidant activity were observed at 500 MPa for 5 min whereas after 60 days of storage the treatments above 300 MPa for 5 min resulted in higher antioxidant capacity indicating the effectiveness of HHP treatments for the production of functional compounds based on gooseberry pulp.

An unexpected result was that reported for evaluation of total antioxidant activity. Percentages of inhibition of the ABTS radical oscillated between 26 and 33% whereas for the hydroxyl-radical scavenging activity ranged between 31 and 38%, independently of the extraction conditions or seaweed solid/liquid ratio used in the extraction process (Table 6.1). Surprisingly, these values are lower than those observed for HWE and UAE extracts from *S. muticum* (Chapter 5, section 5.3.3.). Since a higher concentration of sulphated sugars was observed in HHP-assisted extracts higher antioxidant activities were likewise expected. Despite this non-relationship, the results in Table 6.1 imply that the activity of *S. muticum* extracts still have an important scavenging power on hydroxyl radicals. According to Wijesekara et al. (2011) sulphated polysaccharides not only function as dietary fibre but also contribute to antioxidant activity of seaweeds; heterofucans from *Sargassum filipendula* displayed considerable antioxidant activity expressed as 90.7 ascorbic acid equivalent (Costa et al., 2011). In addition no antidiabetic activity was observed for any of the *S. muticum* extracts obtained by this technique.

6.3.2. Model fitting for HHP-assisted extraction

The results of the 17 experiments including five replicates at the centre point generated from three important parameters, extraction time (X_1), extraction pressure (X_2) and seaweed solid/liquid ratio (X_3) in the Box-Behnken design are listed in Table 6.1 along with the predicted values, within the limits of the experimental factors, by the response surface regression. The second order quadratic models expressed as a function of time, pressure and seaweed solid/liquid ratio are displayed in Tables 6.2 to 6.4 for each measured response, namely, extraction yield, total sugar content, total sulphated sugars content, total antioxidant capacity and hydroxyl-radical scavenging activity.

Table 6.2. Analysis of variance (ANOVA) for the response surface quadratic model for extraction yield of HHP *S. muticum* extracts as a function of the independent variables.

Source	Response Surface Regression					Analysis of Variance			
	Coefficients	Standard errors	*T*-value	R^2	AdjR^2	Degree of Freedom	Mean square	*F*-value	*P*-value
Model	46.6789	1.43713	32.481	99.55%	98.97%	9	11.8093	171.99	0.000
X_1	0.0433	0.04593	0.943			1	0.0611	0.89	0.377
X_2	-0.0162	0.00545	-2.966			1	0.6039	8.80	0.021
X_3	-2.8272	0.29304	-9.648			1	6.3912	93.08	0.000
X_1^2	-0.0016	0.00082	-1.937			1	0.2577	3.75	0.094
X_2^2	0.0000	0.00001	2.195			1	0.3307	4.82	0.064
X_3^2	-0.0745	0.03193	-2.335			1	0.3742	5.45	0.052
X_1X_2	-0.0002	0.00007	-2.168			1	0.3229	4.70	0.067
X_1X_3	0.0195	0.00524	3.729			1	0.9547	13.90	0.007
X_2X_3	0.0026	0.00044	5.876			1	2.3707	34.53	0.001
Residual						7	0.0688		
Lack of fit						3	0.0782	1.27	0.397
Pure error						4	0.0615		
Total						16			

X1= Time (min); X2= Pressure (MPa); X3= Mass (g)

Table 6.3. Analysis of variance (ANOVA) for the response surface quadratic model for total sugar and sulphated sugar contents of HHP *S. muticum* extracts as a function of the independent variables.

Source	Response Surface Regression					Analysis of Variance			
	Coefficients	Standard errors	T value	R^2	AdjR^2	Degree of Freedom	Mean square	*F*-value	*P*-value
Total sugars									
Model	108.736	1.96162	55.432	99.92%	99.82%	9	126.481	988.69	0.000
X_1	-0.253	0.06269	-4.040			1	2.088	16.32	0.005
X_2	0.087	0.00744	11.763			1	17.702	138.38	0.000
X_3	9.686	0.39999	24.216			1	75.021	586.43	0.000
X_1^2	0.044	0.00112	39.141			1	195.985	1531.99	0.000
X_2^2	0.000	0.00001	11.890			1	18.085	141.37	0.000
X_3^2	0.024	0.04358	0.559			1	0.040	0.31	0.593
X_1X_2	-0.003	0.00010	-28.937			1	107.122	837.36	0.000
X_1X_3	0.096	0.00715	13.420			1	23.040	180.10	0.000
X_2X_3	-0.024	0.00060	-40.820			1	213.160	1666.24	0.000
Residual						7	0.128		
Lack of fit						3	0.179	2.00	0.256
Pure error						4	0.089		
Total						16			
Sulphated sugars									
Model	57.1716	1.13591	50.331	99.82%	99.58%	9	18.2576	425.62	0.000
X_1	-0.6465	0.03630	-17.809			1	13.6053	317.17	0.000
X_2	0.0001	0.00431	0.014			1	0.0000	0.00	0.990
X_3	-3.9554	0.23162	-17.077			1	12.5097	291.63	0.000
X_1^2	-0.0106	0.00065	-16.384			1	11.5153	268.44	0.000
X_2^2	-0.0001	0.00000	-16.632			1	11.8661	276.62	0.000
X_3^2	0.0509	0.02523	2.019			1	0.1748	4.07	0.083
X_1X_2	0.0022	0.00006	40.678			1	70.9806	1654.70	0.000
X_1X_3	-0.0448	0.00414	-10.815			1	5.0176	116.97	0.000
X_2X_3	0.0085	0.00035	24.576			1	25.9081	603.97	0.000
Residual						7	0.0429		
Lack of fit						3	0.0774	4.55	0.089
Pure error						4	0.0170		
Total						16			

X1= Time (min); X2= Pressure (MPa); Mass (g)

Table 6.4. Analysis of variance (ANOVA) for the response surface quadratic model for total antioxidant capacity ($ABTS^{\bullet+\cdot}$) and hydroxyl-radical scavenging activity ($OH^{\cdot}$) of HHP *S. muticum* extracts as a function of the independent variables.

Source	Response Surface Regression					Analysis of Variance			
	Coefficients	Standard errors	*T* value	R^2	AdjR^2	Degree of Freedom	Mean square	*F*-value	*P*-value
$ABTS^{\bullet+\cdot}$									
Model	35.3158	1.69079	20.887	99.01%	97.75%	9	7.42164	78.09	0.000
X_1	-0.1648	0.05403	-3.049			1	0.88366	9.30	0.019
X_2	0.0186	0.00641	2.896			1	0.79706	8.39	0.023
X_3	-3.3875	0.34476	-9.826			1	9.17554	96.54	0.000
X_1^2	-0.0018	0.00096	-1.885			1	0.33781	3.55	0.101
X_2^2	-0.0000	0.00001	-5.829			1	3.22921	33.98	0.001
X_3^2	0.2561	0.03756	6.817			1	4.41721	46.48	0.000
X_1X_2	0.0003	0.00008	3.892			1	1.44000	15.15	0.006
X_1X_3	0.0056	0.00617	0.908			1	0.07840	0.82	0.394
X_2X_3	0.0013	0.00051	2.579			1	0.63203	6.65	0.037
Residual						7	0.09504		
Lack of fit						3	0.16486	3.86	0.112
Pure error						4	0.04268		
Total						16			
$OH^{\bullet}$									
Model	17.8751	4.40757	4.056	94.34%	87.07%	9	8.3786	12.97	0.001
X_1	0.2091	0.14085	1.485			1	1.4240	2.20	0.181
X_2	0.0441	0.01671	2.640			1	4.5023	6.97	0.033
X_3	3.3403	0.89873	3.717			1	8.9214	13.81	0.007
X_1^2	-0.0081	0.00251	-3.230			1	6.7395	10.43	0.014
X_2^2	-0.0001	0.00002	-3.152			1	6.4186	9.94	0.016
X_3^2	-0.4290	0.09791	-4.382			1	12.3999	19.20	0.003
X_1X_2	0.0005	0.00021	2.516			1	4.0894	6.33	0.040
X_1X_3	-0.0106	0.01607	-0.661			1	0.2822	0.44	0.530
X_2X_3	0.0002	0.00134	0.173			1	0.0193	0.03	0.868
Residual						7	0.6459		
Lack of fit						3	1.2152	5.55	0.066
Pure error						4	0.2189		
Total						16			

X1= Time (min); X2= Pressure (MPa); Mass (g)

The goodness of fit of the models can be checked by the coefficients R^2 and adj R^2. R^2 values close to 1.0 implies better accuracy of the model, however according to Garai & Kumar (2013) the incorporation of a large number of insignificant variables in the model may result in high R^2 value, but the model may not be able to predict adequate responses. Therefore, the term adj R^2 (which corrects R^2) should also be considered and must be ideally close to R^2. The predictive model for extraction yield, total sugar and total sulphated sugar contents, total antioxidant capacity and hydroxyl-radical scavenging activity had a coefficient R^2 of 0.996, 0.999, 0.998, 0.990 and 0.943, respectively (Tables 6.2-6.4). High values of R^2 with close values of adj R^2, were observed except for hydroxyl-radical scavenging activity (Tables 6.2-6.4), evidenced good correlation between experimental and predicted values with more than 94% variability of the responses explained by the model. Statistical significance and adequacy of the model was tested by analysis of variance and results are shown in Tables 6.2-6.4. All quadratic models used in the experiments were found to be highly significant, as evident from Fisher's F-test with high values of calculated F-values and very low probability values ($p \leq 0.001$). In addition lack of fit was found to be non-significant ($p > 0.05$) suggesting that the model equation for each measured response was adequate to predict the respective values under any sets of combination within the range of experimental variables (Tables 6.2-6.4).

6.3.3. Effect of time, pressure and seaweed solid/liquid ratio on the *S. muticum* extracts analysed responses and optima values

Extraction yield was significantly affected ($p < 0.05$) by pressure (X_2) and seaweed solid/liquid ratio (X_3), interaction effect of time and seaweed solid/liquid ratio (X_1X_3) as well of pressure and seaweed solid/liquid ratio (X_2X_3) (Table 6.2). Results showed that the extraction yield ranged from 32 to 40 g/100g dry seaweed and the highest values (40 g/100g DW) occurred at 5 min (X_1), 450 MPa (X_2), 1g (X_3) and at 17.5 min (X_1) 300 MPa (X_2), 1g (X_3). The 3D response surface plots and 2D contour plots are the graphical representations of regression model, helping to understand the main and the interaction effects between the independent variables (measured responses). The 3D response surfaces for extraction yield are shown in Figures 6.1a-c, respectively.

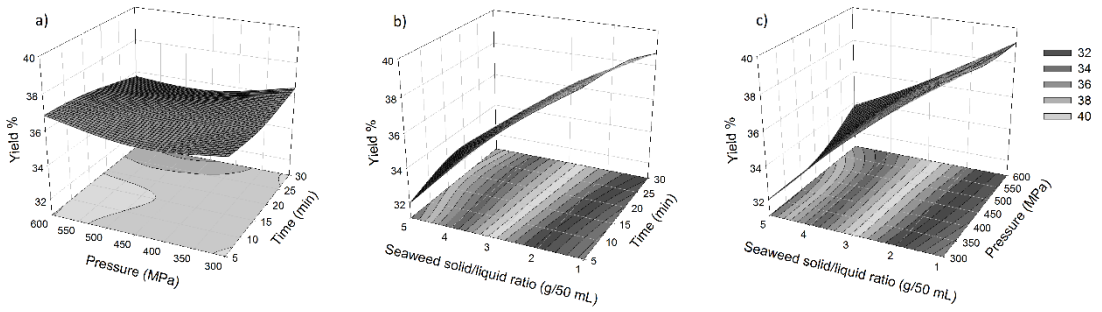

Figure 6.1. Response surface and contour plots showing the effect of two independent variables: pressure and time (a), seaweed solid/liquid ratio and time (b) and seaweed solid/liquid ratio and pressure (c) on the extraction yield for the HHP extraction of *S. muticum*.

Extraction yield decreased with increased seaweed solid/liquid ratio (Figures 6.1b and c; also reflected in the negative value of the β-coefficient value of the linear term) which could be due to the higher ratio of solvent in relation to seaweed mass. No clear effect is visualized for time and pressure, although this last parameter was statistically significant (p=0.021). Indeed, pressure was expected to be one of the most significant extraction factors affecting response variables as reported by other authors in plant extractions (Xi & Wang, 2013), due to its capacity to destroy cell structures and membranes which greatly facilitates mass transfer of solvents into raw materials and the soluble constituents into the solvents (Huang et al., 2013). However, this behaviour will depend on the type of cells; 490 MPa and a liquid/solid ratio of 20mL/g were able to extract maximum phenolic content from green tea (Xi & Wang, 2013). The pattern of Figures 6.1b and c are commonly known as stationary ridge pattern, since the extraction yield did not changed considerably with extraction time (Figure 6.1b) and with the extraction pressure (Figure 6.1c). The maximum predicted extraction yield could be achieved with the following HHP conditions: extraction time (X_1), 5.5 min; extraction pressure (X_2), 300 MPa; and seaweed solid/liquid ratio (X_3):1 g/50 mL (Table 6.5). Under these optima conditions the predictive response for extraction yield was of 40.9 g/100g dry seaweed. The use of HHP assisted extraction has shown great advantages in terms of extraction yields; higher extraction yields with high phenolic contents was reported by Prasad et al. (2010) in comparison to conventional extraction (CE) techniques. Shorter extraction times and higher extraction yields of bioactive compounds are reported by Briones-Labarca et al. (2015) by HHP assisted extraction than by UAE and CE.

Table 6.5. Optima extraction conditions and predictive values for each measured response

Responses	X_1 (Time, min)	X_2 (Pressure, MPa)	X_3 (Mass, g/50 mL)	Predictive value
Yield (g/100g dry seaweed)	5.5	300	1	40.9
Total Sugar ($mg_{glucose\ equiv}/g_{lyoph\ extract}$)	5	600	1	181.5
Total sulfated Sugars ($mg_{Na2SO4\ acid\ equiv}/g_{lyoph\ extract}$)	5	300	1	48.8
Total antioxidant capacity (% scavenging)	5	300	1	34.3
Hydroxyl radical scavenging activity (% scavenging)	29	552	3.7	39.2

For total sugars, all linear, quadratic and interaction effects were significantly affected (p<0.05), except for the quadratic term of seaweed solid/liquid ratio (X_3^2). The response surface plots in Figures 6.2a-c show that total sugar content increased with increased pressure and time (in agreement with the positive values of the corresponding linear terms). The increase of total sugar content (>170$\mu g_{total\ sugar}$/ mg $_{lyophilized\ extract}$) is mostly evident with the variable pressure (Figures 6.2a and 2c) for values higher than 450 MPa. The influence of seaweed solid/liquid ratio is more complex and dependent of the independent variables; in Figure 6.2b the amount of total sugars fluctuated with time but decrease with seaweed solid/liquid ratio whereas in Figure 6.2c an increase is observable for both seaweed solid/liquid ratio and pressure. It is clear that significant interactions occurred and influenced the amount of total sugars extracted. According to the model, the maximum predicted total sugar content could be achieved with the following HHP conditions: extraction time (X_1), 5.0 min; extraction pressure (X_2), 600 MPa; and seaweed solid/liquid ratio (X_3), 1 g/50 mL (Table 6.5). Under these optima conditions the predictive response for yield extraction was of 181.5 $mg_{glucose\ equiv}/g_{lyoph\ extract}$. The amount of sulphated sugars was significantly affected (p<0.05) by extraction time (X1), seaweed solid/liquid ratio, quadratic terms of extraction time and pressure (X_1^2, X_2^2) and all interactions. No significant influence by the extraction pressure was observed with highest values occurring at 300 MPa (Figures 6.2d and f). The lowest pressure and lowest extraction time was enough to extract the maximum of sulphated sugars which is in accordance to the maximum predicted values at extraction time (X_1), 5.0 min; extraction pressure (X_2), 300 MPa; and seaweed solid/liquid ratio (X_3), 1 g/50 mL (Table 6.5). Under these optima conditions the predictive response for extraction yield was of 48.8 $mg_{Na2SO4\ acid\ equiv}/g_{lyoph\ extract}$.

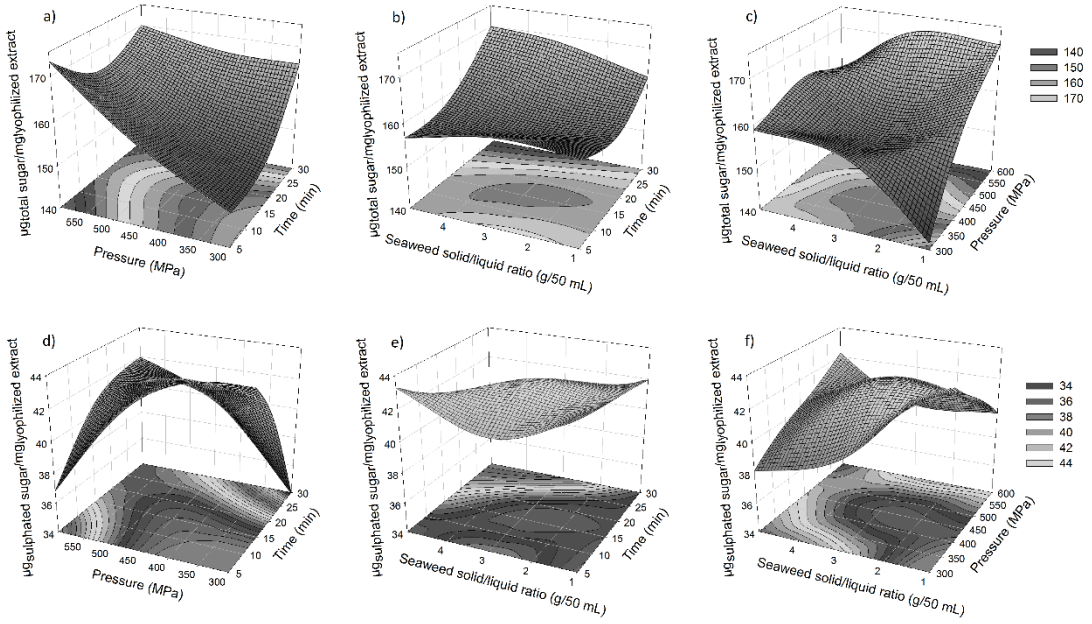

Figure 6.2. Response surface and contour plots showing the effect of two independent variables: pressure and time (a,d), seaweed solid/liquid ratio and time (b,e) and seaweed solid/liquid ratio and pressure (c,f) on the total sugars (a-c) and on total sulphated sugars (d-f) for the HHP extraction of *S. muticum*.

A pressure level of 300 MPa seems to be enough to cause rupture of *S. muticum* cells extracting soluble compounds such as sulphated sugars enabling maximum extraction yield; this happens when compression level exceeds the deformation limit of the cells leading for example to formation of cracks (Huang et al., 2013). According to Kim et al. (2014), HHP assisted extraction with 100 to 300 MPa was not able to increase recovery of solids, polyphenols, total sugars and reducing sugars in the extracts of cactus cladodes (*Opuntia humifusa* Raf.)

In terms of total antioxidant capacity all linear, quadratic and interaction effects were significantly affected (p<0.05) with exception of the quadratic term of extraction time (X_1^2) and interaction of extraction time and seaweed solid/liquid ratio. Results showed that the total antioxidant capacity values ranged from 26 to 33 percentages of scavenging, decreasing with increased seaweed solid/liquid ratio (Figures 6.3b and c) and varying with extraction pressure and time. The maximum values for total antioxidant capacity was observed under the experimental conditions of 5min (X_1), 450 MPa (X_2), 1g (X_3) and 17.5 min (X_1) 300 MPa (X_2), 1g (X_3) which are the same conditions as those that enabled achievement of the highest extraction yield values. Similar optima values as those for extraction yield and sulphated sugars were predicted by the model, i.e. extraction time (X_1), 5.0 min; extraction pressure (X_2), 300 MPa; and seaweed solid/liquid ratio (X_3), 1 g/50 mL (Table 6.5). Under these optima conditions the predictive response for total antioxidant capacity was of 34.3 percentage of scavenging.

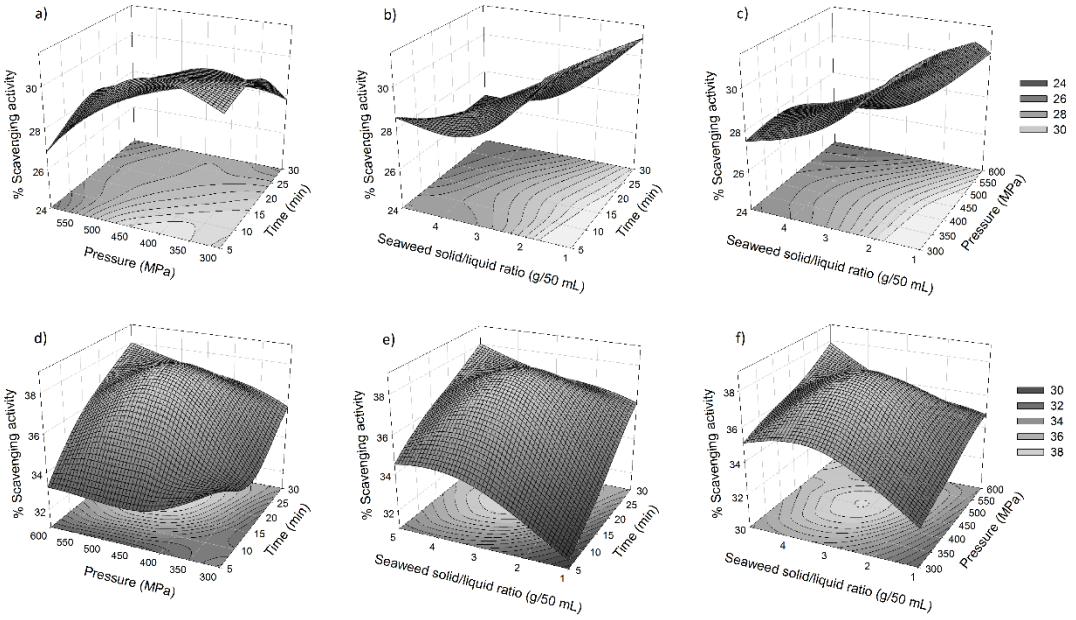

Figure 6.3. Response surface and contour plots showing the effect of two independent variables: pressure and time (a,d), seaweed solid/liquid ratio and time (b,e) and seaweed solid/liquid ratio and pressure (c,f) on the total antioxidant capacity (a-c) and on hydroxyl radical scavenging activity (d-f) for the HHP extraction of *S. muticum*.

Hydroxyl radical scavenging activity was significantly affected (p<0.05) by pressure (X_2) and seaweed solid/liquid ratio (X_3) as well as by the quadratic terms of time, pressure and solid/liquid ratio and also by the interaction effect of extraction time and pressure. In all 3D response surface plots (Figures 6.3d and f) the interaction effects between the independent variables are visible evidenced by the fluctuation of hydroxyl radical scavenging activity along the different axis; Higher values of hydroxyl radical scavenging activity were observed between 3 and 4 g/50 mL, pressure higher than 450 MPa and a longer period of extraction time (> 20 min.) According to the model, the maximum predicted hydroxyl radical scavenging activity could be achieved with the following HHP conditions: extraction time (X_1), 29.0 min; extraction pressure (X_2), 552 MPa; and seaweed solid/liquid ratio (X_3), 3.66 g/50 mL (Table 6.5). Under these optima conditions the predictive response for hydroxyl radical scavenging activity was of 39.2 percentage of scavenging**.**

6.4. Conclusions

In this chapter, response surface methodology was applied for optimizing HHP assisted extraction of bioactive polysaccharides from *S. muticum*. This technology improved extractability (higher extraction yields) and bioactivity from brown seaweeds such as *S. muticum* providing extracts concentrated in polysaccharides which can be explored as an ingredient source in the development of novel functional foods. The maximum values of all responses were determined under different

optimum conditions of HHP processing: 5-5.5 min, 300 MPa and 1 g/50 mL of dry seaweed for extraction yield, sulphated sugar content and total antioxidant capacity; same conditions but with 600 MPa for total sugar content and 29 min, 552 MPa and 3.7 g of dry seaweed for hydroxyl-radical scavenging activity. Overall, the results demonstrated that the minimum processing tested values for extraction time, extraction pressure and seaweed solid/liquid ratio are a set of experimental conditions that enable achieving maximum values for three of the important measured responses namely higher extraction yields, sulphated sugar content and total antioxidant capacity (well correlated between each other) which demonstrate the potential efficiency of HHP to extract compounds from brown seaweeds such as *S. muticum*, an invasive seaweed, in a few minutes. The experimental conditions allow a fast and cost-saving process in extraction of bioactive polysaccharides.

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Chapter 7

Impact of enzyme and ultrasound assisted extraction methods on cultivated *Pholiota nameko* and its biological properties

7.1. Introduction

As previously discussed edible mushrooms are a valuable source of nutrients and of bioactive compounds with different biological properties such as antioxidant, antitumor, immunomodulation, anti-diabetic, anti-inflammatory, cardiovascular disease preventive and anti-cancer roles (El Enshasy & Hatti-Kaul, 2014; Zhang et al., 2014; Zhu et al., 2014; Ma et al., 2013) representing a potential sources of functional ingredients. *Pholiota nameko, a* wood-rotting fungus widely cultivated in China and Japan (Li et al., 2012) but much less in Europe, it is known for its pleasant flavour being very popular in many dishes. Its biological properties have been studied especially in terms of anti-inflammatory, antioxidant and immunomodulatory activities (Li et al., 2014; Li et al., 2008a,b). A novel antitumor protein from *Ph. nameko* has recently been reported by Zhang et al. (2014). Due to its biological potential, this edible and cultivable mushroom was selected from those studied and characterized in chapter 4 to undergo different extraction methods. The main objective of the study in this chapter was to obtain extracts rich in bioactive compounds using alternative water-based approaches described in chapter 5 such as enzyme assisted extraction (EAE), ultrasound assisted extraction (UAE) and hot water extraction (HWE. Hence, this study was based on 3 steps: i) performance of different extraction modes and evaluation of respective yield; ii) chemical characterization of each extract obtained from each extraction mode and, iii) evaluation of the biological potential of the extracts in terms of antioxidant, anti-diabetic and prebiotic activities.

To my knowledge, this is the first study applying water-based extraction through combined EAE and UAE on cultivated *Ph. nameko* evaluating chemical and biological properties of the different extracts in order to assess their potential added value to be used in food and/or nutraceutical applications.

7.2. Material and methods

7.2.1. Selected mushroom species

As previously described specimens of *Pholiota nameko* were selected to be studied in this chapter. The mushroom cultivation, dryness and grinding was performed according procedures described in chapter 4, section 4.2.1.

7.2.2. Ultrasound and enzymatic-assisted extraction

Ultrasound and enzymatic assisted extractions as well as hot water extraction to *Ph. nameko* were performed according to procedures described in chapter 5, section 5.2.2.

7.2.3. Chemical characterization of the extracts

Chemical characterization of extracts namely the determination of content in moisture, organic matter, ash, nitrogen, total fat, total sugar as well as total polyphenols in the extracts was determined according to procedures described in chapter 3, section 3.2.2.1.

7.2.4. Determination of biological properties of the extracts

The antioxidant capacity of extracts was determined according to procedures described in chapter 5, section 5.2.4 whereas prebiotic potential was determined according to procedures described in chapter 5, section 5.2.5. The α-glucosidase inhibitory activity of the extracts was determined according to procedures described in chapter 5, section 5.2.6.

7.2.5. Statistical analysis

One-way ANOVA was carried out for each measured parameter, except for prebiotic activity, with SigmaStat™ (Systat Software, Chicago, IL, USA) to assess if extraction method was a significant source of variation (p=0.05). The Holm-Sidak method was used for pair-wise comparisons (p=0.05). In terms of prebiotic activity, for each probiotic bacterium, a two-way ANOVA was carried out with SigmaStat™, to assess whether the extract and incubation time were significant sources of variation (p=0.05). Since significant differences were observed for each factor and interactions, one-way ANOVAs were carried out to observe if the source of carbon (glucose, FOS or extract) was statistically significant. One-way ANOVAs were also performed to evaluate statistical significance of the viable cell numbers after 24/48h, in comparison to values at 0h

7.3. Results and Discussion

7.3.1. Extraction yield and chemical characterization of the extracts

The extract yields obtained from water-based extractions of *Ph. nameko* mushroom are listed in Table 7.1. Extraction yields between 54 to 63% were obtained for extracts obtained by EAE with proteases, HWE or UAE. The lowest yield was obtained by EAE with Alcalase whereas the differences between HWE, UAE and EAE with Flavourzyme were not significantly different (p>0.05). Cellulase- and Viscozyme-assisted extractions were responsible for the statistically significant (p<0.05) highest extraction yields in *Ph. nameko* (67-77%). The extraction of more compounds from the mushroom cell structure was reflected in the statistically significant higher solid content (p<0.05) in their lyophilized extracts in comparison to all other extracts. Being fungal cell walls constituted mostly by polysaccharides that comprise about 80% of their dry weight (Ruiz-Herrera, 1991), it is expected that carbohydrases hydrolyse cell wall components thereby increasing their permeability and resulting in higher extraction yields as stated previously by Puri et al. (2012). According to my best of knowledge only a few studies are available with EAE on

mushrooms but none applied to *Ph. nameko*. Yin et al. (2011) carried out an optimization of polysaccharides extraction from *Tricholoma matsutake* using EAE with a mixture of dual enzyme activities – proteases and carbohydrases (papain, pectinase and cellulase, ratio 1:1:1) testing several parameters but no comparisons were made with other extraction modes. Zhu et al. (2014) also carried out an optimization of polysaccharides extraction from *Hericium erinaceus* using EAE with a mixture of enzymes (Cellulase, Pectinases and Trypsinase, ratio 2:2:1); the optimal extraction conditions reported resulted in a higher yield (increase of 67.7%) in comparison to those obtained by HWE. In this study, lower extraction yields have been achieved using a single enzyme activity. However, they are interesting from the standpoint of the possibility of using different enzymes combination to improve extraction yield.

Table 7.1. Extraction yields and contents of nitrogen, sugars, and total phenolics in the different extracts of *Ph. nameko*.

Extraction method	Extraction yields ($g_{lyoph\ extract}/100g_{dry\ mushroom}$)	Nitrogen ($mg/g_{lyoph\ extract}$)	Sugars ($mg_{glucose\ equiv}/g_{lyoph\ extract}$)	Total phenolic content ($\mu g_{cathecol\ equiv}//g_{lyoph\ extract}$)
HWE	62 ± 1^{b}	43.7 ± 0.3^{b}	527 ± 32^{b}	153 ± 1^{ab}
UAE	62.86 ± 0.07^{b}	44.0 ± 0.4^{b}	628 ± 33^{c}	144 ± 3^{a}
EAE_Cell	76.8 ± 0.6^{d}	39.5 ± 0.4^{a}	574 ± 20^{bc}	153 ± 13^{a}
EAE_Visc	67.1 ± 0.4^{c}	40.3 ± 0.5^{a}	532 ± 12^{b}	164 ± 9^{ab}
EAE_Alc	54.5 ± 0.7^{a}	51.8 ± 0.5^{d}	376 ± 36^{a}	158 ± 8^{ab}
EAE_Flav	61 ± 2^{b}	46.6 ± 0.3^{c}	526 ± 25^{b}	182 ± 9^{b}

For each parameter, different letters indicate significant differences (p<0.05) between methods of extraction.

Ultrasound assisted extraction on *Ph. nameko* did not positively affect yield extraction in comparison to HWE. Variable tendencies have been reported for other mushroom polysaccharides yield extraction by UAE which is dependent on mushroom species but also on technological parameters such as ultrasonic power, temperature, time and ratio or water volume to dry biomass (Cheung et al., 2012; Tian et al., 2012). Higher polysaccharides yield than those reported in this study were obtained with *Lentinus edodes* and *Agaricus bisporus* but similar or lower extraction yields were reported for *Grifola frondosa* and *Coriolus versicolor,* respectively (Cheung et al., 2012; Tian et al., 2012). Physical properties of different mushroom species could be in part responsible for different effects of UAE on the efficiency of polysaccharides extraction.

In general, and as expected, EA extracts obtained with proteases action presented higher nitrogen content (Table 7.1); Alcalase and Flavourzyme were responsible for the highest content namely 52 mg $N/g_{lyoph\ extract}$ and 47 mg $N/g_{lyoph\ extract}$, respectively. These values correspond to 73-74% of nitrogen content present in the dry mushroom which was 38.6 $mg/g_{dry\ mushroom}$ (Chapter 4, Table 4.2). Indeed, proteases were the most effective enzymes for protein recovery and accessibility highlighting the Alcalase endopeptidase activity which resulted in a slightly higher recovery rate.

In terms of sugar content, UAE extracts presented the highest content with 628 $mg_{glucose\ equiv}/g_{lyoph\ extract}$, followed by EA extracts obtained from carbohydrases action responsible for values of 574 and 531 $mg_{glucose\ equiv}/g_{lyoph\ extract}$ in Cellulase and Viscozyme extracts, respectively. The highest sugar content in UAE extract may result from the mechanical action of ultrasound waves promoting a higher breakdown and release of mushroom cell wall polysaccharides. It must be emphasized that Cellulase was able to extract 74% of total sugars present in the dry mushroom which was 596 $mg/g_{dry\ mushroom}$ (Chapter 4, Table 4.2) whereas 55, 66 and 60% of total sugars were extracted by HWE, UAE and EAE with Viscozyme, respectively. Alcalase EA extracts had the statistically significant lowest sugar content being able to extract only 34% of total sugars present in dry mushroom.

Total phenolic content varied slightly with highest values observed in EA extracts with Viscozyme, Alcalase and Flavourzyme; values ranged between 158 to 182 $\mu g_{cathecol\ equivalent}/g_{lyoph\ extract}$ (Table 7.1), corresponding to extraction efficiency of 13-16% of total phenolics present in dry mushroom (Chapter 4, Table 4.2). The use of cell wall degrading enzymes as alternative extractive auxiliaries provided the better access to the *Ph. nameko* derived phenolic compounds. Nevertheless the relatively lower extraction values can be expected because it is known that other organic solvents such as ethanol may be more efficient for polyphenol extraction than water (Farvin & Jacobsen, 2013). On the other hand ethanol based extraction may not provide the same versatility for food applications as water based extraction and reports have shown the antioxidant potential of *Ph. nameko* water extracts. Wang & Xu (2014) evaluated the total phenolic content of 20 different edible mushroom species including *Ph. nameko* using acetone, ethanol, water and hot water as extraction solvents. Water extracts reported the highest values (2.15 and 7.31 $mg_{gallic\ acid\ equivalent}/g_{dry\ mushroom}$). However, the authors determined the total phenolic content applying the Folin-Ciocalteu method directly to the water extracts which could easily overestimate the phenolic content due to chemical interferences. In addition, the chemical composition of saprophytic mushrooms is directly influenced by the organic substrate used for their growth either in a controlled or in wild environments which can lead to variable content of second metabolites such as phenolics.

7.3.2. Antioxidant activity of the extracts

Total antioxidant capacity and hydroxyl radical scavenging activity were determined and data are presented in Figures 7.1a-b. All the different lyophilized extracts were able to scavenge $ABTS^{\bullet+}$ radical. At the concentration of 2 $mg_{lyoph\ extract}$/mL 35 to 44% scavenging activity was observed for all extracts. Enzymatic extracts obtained with proteases registered the highest total antioxidant capacity with 41- 44% scavenging activity. Higher concentrated extracts were tested and IC_{50} values of 2.5-2.6 $mg_{lyoph\ extract}$/mL were obtained for enzymatic extracts with the proteases, whereas 3.1-3.4 $mg_{lyoph\ extract}$/mL were needed for the other enzymatic extracts as well as for HWE and UAE extracts.

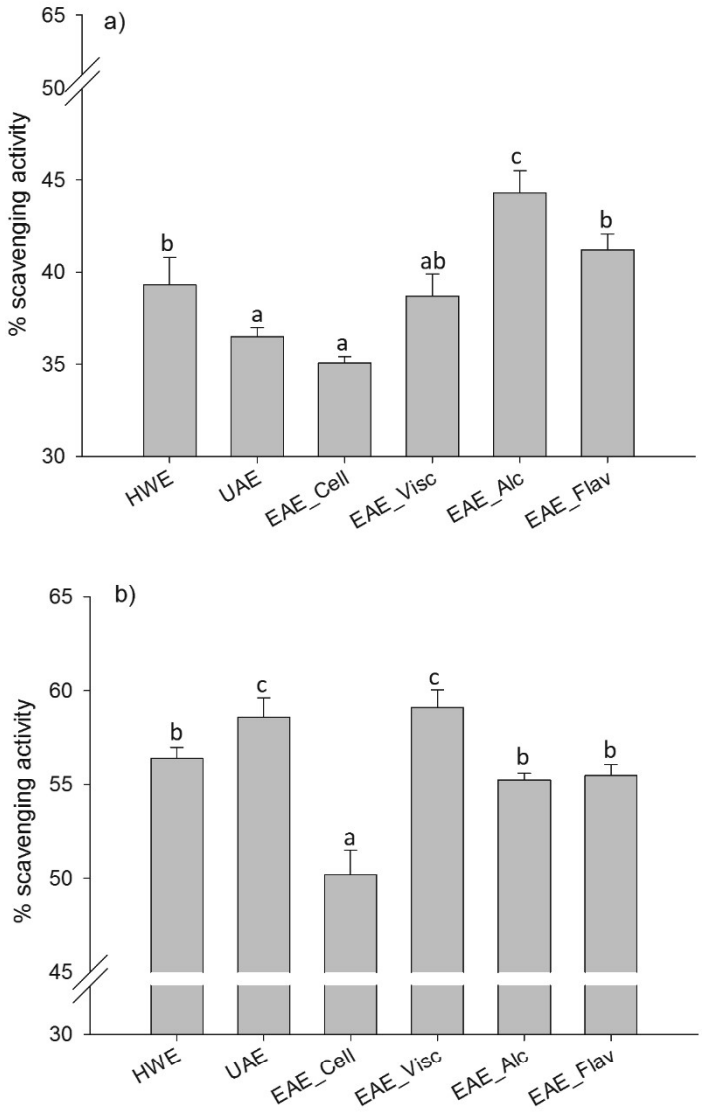

Figure 7.1. Total antioxidant capacity (a) and hydroxyl radical scavenging activity (b) of *Ph. nameko* extracts obtained by hot water extraction (HWE), ultrasound-assisted extraction (UAE) and enzymatic-assisted extraction (EAE) with Cellulase (Cell), Viscozyme (Visc), Alcalase (Alc) and Flavourzyme (Flav) at 50 ºC. Different letters indicate significant differences (p<0.05) between extracts.

Potential antioxidant activity in water and hot water extracts from *Ph. nameko* were reported by Ji et al. (2012) and Wang & Xu (2014); in both studies, a higher scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was reported for water extracts than for ethanol extracts.

In general, a good scavenging capacity for $HO^{\bullet}$ reactive oxygen species was obtained, in general, for all 2 $mg_{lyoph\ extract}$/mL water extracts (Figure 7.1b). More than 55% scavenging activity was observed with HWE, UAE and EA extract with Viscozyme. For the other extracts the scavenging activity was between 50-55%. The Cellulase EAE had the statistically significant lowest scavenging activity for both the $ABTS^{\bullet+}$ and $OH^{\bullet}$ radicals (p<0.05). This extract was characterized by the highest extraction yield, rich in sugars but with lower values of nitrogen and total phenolic

content (Table 7.1). Although significant correlations are not possible, it seems that the statistically significant difference between the extracts (p<0.05), could be, at least in part, attributed to total phenolic compounds as well as to protein compounds present in *Ph. nameko* which may eventually indicate that some protein degradation products may be related with the antioxidant properties. A positive correlation between antioxidant capacity and total phenolic content in several mushrooms species has also been reported by Guo et al. (2012). More recently, a novel antitumor protein was isolated from *Ph. nameko* exhibiting significant antioxidant activity by effectively scavenging $HO^{\bullet}$ and DPPH radicals in comparison to standard antioxidant butylated hydroxyl anisole (Zhang et al. 2014).

7.3.3. Prebiotic potential of mushroom extracts

Mushrooms are a potential source of prebiotics because they are rich in non-digestible dietary fibres such as glucans, chitin and heteropolysaccharides (Bhakta & Kumar, 2013) which could play beneficial and prebiotic roles. Nonetheless, to my best of knowledge, no information is available regarding the prebiotic potential of the *Ph. nameko* or of its extracts. Hence, the potential prebiotic effect of different *Ph. nameko* extracts was evaluated against *L. acidophilus* La-5 and of *B. animalis* BB12 strains, the usual target genera for prebiotics, and results are reported in Table 7.2. In general, we were able to observe a prebiotic potential of *Ph. nameko* extracts upon growth of both probiotic strains, when used as the single carbon source. Moreover, mushroom extracts were able to increase growth similarly to that promoted by traditional carbon source (glucose) as well as promote longer survival stability.

Significantly higher viable numbers (p<0.05) of *L. acidophilus* La-5 were observed for all the extracts after 24/48h of incubation in comparison to growth in glucose or in FOS; associated higher growth rates were also observed (p<0.05). An increase of 2 log cycles was observed with HWE, UAE as well as with EA assisted by Viscozyme and Flavourzyme (Table 7.2) resulting in incremental ratios of 1.3 after 48h. Slightly lower viable cell numbers of *L. acidophilus* La-5 were observed in EAE with Alcalase and Cellulase especially after 48h of incubation resulting in incremental ratios of 1.25-1.23.

Table 7.2. Mean and standard deviation of cell counts [Log (cfu/mL)] of *L. acidophilus* La-5 and *B. animalis* BB-12 throughout 48h of incubation at 37 ºC in the presence or absence of glucose, with FOS or with mushroom extract.

	Time (hrs)	No Sugar		Glucose		FOS		Time (hrs)	HWE		UAE		EAE_Visc		EAE_Cell		EAE_Alc		EAE_Flv	
		Log (cfu/mL)	R*	Log (cfu/mL)	R*	Log (cfu/mL)	R*		Log (cfu/mL)	R*	Log (cfu/mL)	R*	Log (cfu/mL)	R*	Log (cfu/mL)	R*	Log (cfu/mL)	R*	Log (cfu/mL)	R*
L. acidophilus La-5	0	7.00±0.08	1.00	7.00±0.08	1.00	7.00±0.08	1.00	0	7.00±0.08	1.00	7.00±0.08	1.00	7.00±0.08	1.00	7.00±0.08	1.00	7.00±0.08	1.00	7.00±0.08	1.00
	4	7.41±0.04	1.06	7.57±0.06	1.08	7.50±0.05	1.07	4	7.6±0.2	1.09	7.72±0.04	1.10	7.6±0.1	1.08	7.62±0.01	1.09	7.48±0.03	1.07	7.55±0.02	1.08
	8	7.92±0.01	1.13	8.11±0.02	1.16	8.0±0.1	1.15	8	8.8±0.1	1.25	8.67±0.04	1.24	8.74±0.09	1.25	8.44±0.08	1.21	8.36±0.02	1.19	8.48±0.03	1.21
	12	5.81±0.01	0.83	5.8±0.4	0.83	7.56±0.01	1.08	12	8.9±0.3	1.27	9.01±0.06	1.29	9.04±0.01	1.29	8.97±0.08	1.28	9.04±0.05	1.29	8.95±0.03	1.28
	24	<3.7[a]	-	3.94±0.07[c]	0.56	5.9±0.2[b]	0.84	24	**9.26±0.01*[d]**	**1.32**	**9.13±0.01*[d]**	**1.30**	**9.22±0.01*[d]**	**1.32**	**9.35±0.04*[d]**	**1.34**	**9.21±0.04*[d]**	**1.32**	**9.24±0.07*[d]**	**1.32**
	48	<3.7[a]	-	4.8±0.2[b]	0.69	6.3±0.8[c]	0.90	48	**9.0±0.2*[e]**	**1.29**	**8.95±0.04*[e]**	**1.28**	**9.05±0.04*[e]**	**1.29**	**8.78±0.01*[ed]**	**1.25**	**8.61±0.06*[d]**	**1.23**	**9.06±0.05*[e]**	**1.29**
B. animalis BB12	0	7.59±0.07	1,00	7.59±0.07	1,00	7.59±0.07	1.00	0	7.59±0.07	1,00	7.59±0.07	1,00	7.59±0.07	1,00	7.59±0.07	1,00	7.59±0.07	1,00	7.59±0.07	1,00
	4	7.3±0.1	0.96	7.8±0.2	1.03	7.58±0.03	1.00	4	7.6±0.1	1.00	7.50±0.03	0.99	7.62±0.02	1.00	7.71±0.01	1.02	7.38±0.04	0.97	7.61±0.01	1.00
	8	7.20±0.05	0.95	8.0±0.3	1.05	8.09±0.07	1.07	8	8.5±0.2	1.12	8.3±0.1	1.09	9.0±0.2	1.18	8.5±0.1	1.12	7.41±0.03	0.98	7.8±0.2	1.03
	12	6.83±0.01	0.90	7.8±0.1	1.03	8.6±0.1	1.13	12	9.08±0.06	1.20	8.95±0.03	1.18	9.3±0.1	1.23	8.79±0.01	1.16	7.68±0.03	1.01	7.9±0.1	1.04
	24	4.5±0.5[a]	0.60	4.9±0.1[a]	0.65	7.8±0.2[bc]	1.03	24	**9.10±0.04*[cd]**	1.20	**9.27±0.02*[cd]**	**1.22**	**9.36±0.01*[d]**	**1.23**	**9.0±0.2*[cd]**	**1.19**	7.4±0.2*[b]	0.98	**8.46±0.01*[c]**	**1.11**
	48	<3.7[a]	0.36	5.0±0.8[b]	0.66	5.49±0.03[b]	0.72	48	**9.17±0.05*[c]**	**1.21**	**9.30±0.01*[c]**	**1.23**	**9.37±0.01*[c]**	**1.23**	**9.0±0.1*[c]**	**1.19**	5.70±0.01*[b]	**0.75**	**8.44±0.03*[c]**	**1.11**

[1]Ratio = (Log N_i)/(Log N_0); N = mean (cfu/mL) at time i=4, 8, 12, 24 or 48 hrs; N_0 = mean (cfu/mL) at time 0 hrs; Bold typing for values higher than those obtained with prebiotic FOS after 24 and 48h and * for significant differences ($p<0.05$) in comparison to values at 0h; Different letters in the same row (24 or 48h) for each probiotic strain indicate significant differences ($p<0.05$) for the viable cells between extracts or controls.

Evidence of a potential prebiotic effect was also observed for *B. animalis* BB12. Viable cell numbers at 24/48h of incubation in all extracts, except for EA with Alcalase (extract with the lowest sugar content), were significantly higher than those obtained with prebiotic FOS and glucose (p<0.05). Similar to *L. acidophilus* La-5, significant increased growth rates (p<0.05) were also observed. Increases of 1.4 to 1.8 log cycles were observed with HWE, UAE as well as with enzymatic extracts assisted by Viscozyme and Cellulase (Table 7.2) resulting in incremental ratios of 1.2 after 48h.

Content and type of sugars present in the extracts are determinant factors for the growth and viability of probiotic bacteria. It is known that cellulose, chitin, β-glucans, α-glucans and glycoproteins are present in the outer or inner layers of fungal cell walls being more or less extractable depending on the extraction method; water-soluble polysaccharides have been extracted with hot water whereas water-insoluble polysaccharides by alkali solutions from mushrooms (Zhang et al., 2007).

Since no studies on prebiotic potential of *Ph. nameko* were found in the literature some pertinent observations with other mushroom species are described. For example, Synytsya et al. (2009) evaluated different extracts of *Pleurotus ostreatus* and *Pleurotus eryngii* for their potential prebiotic activity; specific glucans were isolated by boiling water and alkali extraction and tested using nine probiotic strains of *Lactobacillus*, *Bifidobacterium* and *Enterococcus*. According to these authors, two types of glucans and proteoglucan complexes from *Pleurotus* can be used as synbiotics with selected probiotic strains. Giannenas et al. (2010) evaluated the intestinal morphology and bacteria populations in broiler chickens fed for six weeks diets that contained different amounts of the mushroom *Agaricus bisporus*; an improved performance of broiler chickens was observed when adding 10 and 20 g/kg of the edible mushroom to the broilers' diet with significant increases in the viable cell numbers of *Lactobacillus* spp. in the ileum as well as in *Lactobacillus* spp. and *Bifidobacteria* spp. in the caecum of chickens.

According to the results, the majority of the extracts obtained from *Ph. nameko* possess compounds that can be metabolized by *L. acidophilus* La-5 and *B. animalis* BB12. Higher prebiotic potential was observed for HWE, UAE, and EAE with Viscozyme and Cellulase. Given the reported results in terms of growth promotion of *L. acidophilus* La-5 and *B. animalis* BB12 and considering the prebiotic potential of polysaccharides and extracts from mushrooms reported by literature concerning other species, further tests (non-digestibility and selective fermentation capacity in model systems) must be performed with these specific extracts.

7.3.3. Anti-diabetic activity

Small molecules with α-glucosidase or α-amylase inhibition potential are of pharmaceutical interest as anti-diabetic drugs since by acting as competitive inhibitors of the enzyme they inhibit the hydrolysis of oligosaccharides, trisaccharides and disaccharides to glucose and other monosaccharides in the small intestine and thereby delay postprandial glucose absorption lowering

blood glucose (Ali et al., 2006). All the extracts of *Ph. nameko* showed α-glucosidase inhibitory activity although to different extensions (Figure 7.2). The extracts with the highest inhibitory activity were the UAE (49%) and EAE with Cellulase (50%) whereas the extract with the lowest inhibitory activity was the EAE with Alcalase (14%). To my knowledge, there are no reports on potential anti-diabetic of water-based *Ph. nameko* extracts. Therefore, no direct comparisons with other works are possible only with other mushroom species and other extraction modes. For example, Liu et al. (2012) evaluated the chemical composition and anti-hyperglycemic and antioxidant activity of five wild edible mushrooms from Southwest China. Based on their results, ethanolic and aqueous extracts showed anti-hyperglycemic activity. In particular, the aqueous extract of *Catathelasma ventricosum* revealed the highest α-glucosidase inhibitory activity with an IC50 value of 2.74 μg/mL.

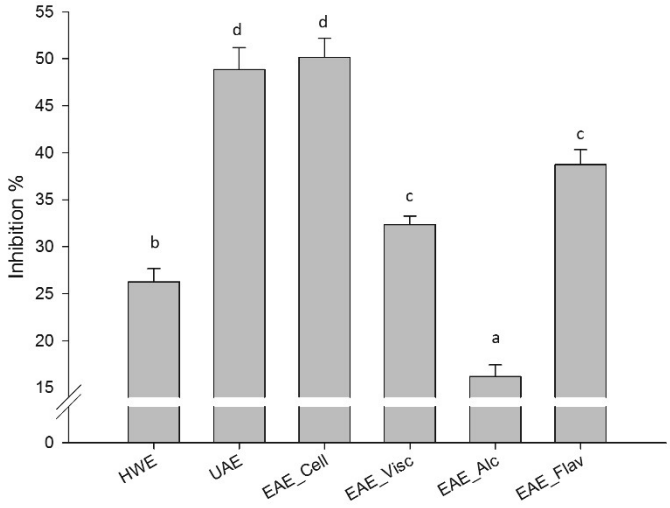

Figure 7.2. α-glucosidase inhibitory activity, expressed as % of inhibition, of *Ph. nameko* extracts obtained by hot water extraction (HWE), ultrasound-assisted extraction (UAE) and enzymatic-assisted extraction (EAE) with Cellulase (Cell), Viscozyme (Visc), Alcalase (Alc) and Flavourzyme (Flav) at 50 ºC. Different letters indicate significant differences (p<0.05) between extracts.

Zhu et al. (2014) reported over 40% of α-glucosidase inhibitory activity from oyster mushroom polysaccharides. Hsu et al., (2013) reported α-glucosidase inhibitory properties by intracellular polysaccharides from *Coriolus versicolor* which were related to the presence of α-(1,4) glycosidic linkages and total relative percentage of D-Glucose and D-Galactose in the polysaccharide structure, other than triterpenoids. In this study UAE and EAE with Cellulase from *Ph. nameko* had the highest contents in total sugars (Table 7.1) which probably evidences the presence of polysaccharides with α-glucosidase inhibitory properties. The use of α-glucosidase inhibitors are an important strategy in the prevention (functional food) or management (therapeutic agent) of blood glucose levels in type 2 diabetic and borderline patients.

7.4. Conclusions

Enzymatic assisted extraction and UAE revealed to be promising tools to obtain extracts from standard cultivated *Ph. nameko* with attractive biological properties. Hydrolytic enzymes were able to improve the extraction yield with carbohydrases extracting more compounds from the cell structure of the mushrooms. However taking into consideration the general results achieved it becomes difficult to pinpoint the most efficient extraction method with the highest biological potential since it depends on the extraction mode considering the duality: extraction yield vs chemical/biological properties and on the type of analysis made. In this perspective, all *Ph. nameko* extracts presented added value in particular due to the interesting prebiotic potential. In addition, promising α-glucosidase inhibitory activity was observed in UAE and EAE with Cellulase and consequently an attractive anti-diabetic potential is proposed. In terms of antioxidant capacity, no strong activity was observed, yet it was of higher potential in UAE and EA with Alcalase and Viscozyme extracts. Therefore, conjugating extraction yield with prebiotic effect and α-glucosidase inhibitory activity, EA extracts resulting from carbohydrases' action are of potential interest where probably the polysaccharides play the major role. For future studies of functional food development with standard cultivated *Ph. nameko*, the selection of the most efficient extraction mode will need to be coupled to the best results in terms of biological properties according to the intended purpose.

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Chapter 8

Chemical and structural characterization of the selected extracts from seaweeds and mushrooms

8.1. Introduction

The main driving force of this thesis is to find in the realm of the seaweeds and mushrooms worlds naturally produced bioactive compounds to be used as alternatives to synthetic substances in the development of functional foods or nutraceuticals to promote healthy effects in the organism. In order to do so an integrated approach was established and the various steps have been followed chronologically. Firstly, a set of seaweeds from the three different classes and a set of edible mushrooms were characterised for their proximate composition and potential bioactive compounds (special attention was paid to phenolic compounds and polysaccharides from both a preservation and bioactive point of view, albeit proteinaceous substances were also considered important); once the characterization was achieved the need for suitable, fast, cost-effective and environmentally friendly extraction methodologies capable of extract the biologically active natural ingredients of interest from both the seaweeds and mushrooms led to the second stage which involved the study of several extraction techniques (including the use of enzymes to assist in extraction) and parameters in order to fully characterize the potential of the different natural sources, introducing different extraction selectivity and efficiency while aiming at maximum preservation of bioactivity. Upon attainment of the different extracts these were then tested for different biological activities using appropriate *in vitro* assays important for the bioactivity screening procedure. Extracts isolated from the selected seaweeds and mushrooms demonstrated important biological properties such as antioxidant, antidiabetic and prebiotic activities, as described in chapters 5 to 7. The present chapter will cover the next stage in this integrated research related with the chemical characterization of the bioactive components present in the four selected extracts from seaweeds and mushrooms where target biological activities were confirmed or more promising (Tables 8.1 and 8.2). In this respect, analytical techniques to be used will depend on the nature of the extract, its stability and the nature of the envisaged bioactive compounds. In many cases several advanced analytical techniques are performed in parallel in order to maximize identification potential. Furthermore, upon chemical and structural characterization correlation with the observed bioactivities is required in order to establish causal relationships responsible for the health-promoting activities.

Hence the objective of the research work presented in this chapter is the chemical and structural identification of the two selected extracts of seaweeds and the two selected extracts of mushrooms, and the consecutive correlation with previously observed bioactivities. Elemental composition as well as the composition in amino acids and monosaccharides was determined for each extract, complementing chemical characterization of the extracts presented in chapters 5 to 7, whereas structural characterization were based on FTIR-ATR and ^{1}H NMR analysis.

8.2. Material and methods

8.2.1. Selected seaweeds and mushroom extracts

The seaweed extracts selected for chemical and structural characterization were the enzymatic extracts of *S. muticum* obtained with Alcalase and of *O. pinnatifida* obtained with Viscozyme. In terms of mushroom extracts, the enzymatic extracts of *Ph. nameko* obtained with Cellulase and with Flavourzyme were the selected choices.

8.2.2. Chemical characterization

8.2.2.1. Elemental Analysis

The determination of the inorganic elements Mo, B, Zn, P, Cd, Co, Ni, Mn, Fe, Mg, Ca, Cu, Na, Al and K in lyophilized extracts was performed in two steps: microwave-assisted digestion followed by determination of the 15 elements using an inductively coupled plasma (ICP) optical emission spectrometer (OES) with radial plasma configuration according to procedures described in chapter 3, section 3.2.2.3 and chapter 4, section 4.2.4. Three replicates were performed for each sample as well as blanks.

Organic elements C, H, S and N in lyophilized extracts were quantified using a Truspec 630-200-200 Elemental Analyser (Mönchengladbach, Germany). Triplicate samples up to 3 mg for each extract were placed under combustion at 1075 ºC. Carbon, H and S were detected by infrared absorption whereas N was detected by thermal conductivity.

8.2.2.2. Analysis of monosaccharides composition

Monosaccharide composition was analysed by high performance liquid chromatography (HPLC) after acid hydrolysis. For each lyophilized extract, 2.5 mg of sample were hydrolysed with 2 mL of 2 M trifluoroacetic acid at 110 °C for 4 h. The hydrolysate was then dried by vacuum evaporation at 50 °C and re-dissolved in 2 mL deionized water. The hydrolysate solution (450 μL) was mixed with 450 μL of 1-phenyl-3-methyl-5-pyrazolone solution (0.5 M in methanol) and 450 μL NaOH solution (0.3 M) and then reacted at 70 °C for 30 min. The reaction was stopped by neutralizing with 450 μL of 0.3 M HCl, and the product was then partitioned with chloroform three times. The aqueous layer was collected and filtered through a 0.45 μM membrane and was applied to HPLC.

The HPLC was performed in an Agilent 1100 (Waldbronn, Germany) and a ZORBAX ECLIPSE XDB-C18 column (4.6 x150 mm, 5 micro) at 25 °C with potassium phosphate buffer saline (0.05 M, pH 6.9) with 15% (solvent A) and 40% acetonitrile (solvent B) as mobile phases and detected by UV detector at 250 nm. All analysis were made in quintuplicate and quantified using a

calibration curve built with monosaccharides standards (Sigma Aldrich, St. Louis MO, USA) and expressed as $mg/g_{lyophilized\ extract}$. D- and L-Glucose, D- and L-galactose, DL-arabinose, L-fucose, D- and L-mannose, D-xylose, L-rhamnose, D-glucuronic acid, D-galacturonic acid and D-glucosamine-6-phosphate were used as the standards. Recovery ranged between 93 and 99 % with a LOD of 0.095 mg/g.

8.2.2.3. Analysis of amino acids

Free amino acids content of each extract was performed by pre-column derivatization with orthophthalaldehyde (OPA) methodology. Isoindole-type fluorescent derivatives were formed in an alkaline solution (borate buffer pH 10.4) from OPA, 2-sulfanylethanol and the primary amine group of the amino acid. The derivatives were separated by HPLC (Beckman coulter, California USA) coupled to a fluorescence detector (Waters, Milford. MA, USA) according to the procedure of Proestos et al. (2008). 100 µL of each sample, at concentration 10 mg mL^{-1}, was derivatized according to the OPA method and injection volume of derivatives was 20 µL. All analysis were made in triplicate and quantified using a calibration curve built with amino acids pure standards (Sigma Aldrich, St. Louis MO, USA) and expressed as g/100 g of protein content. Recovery ranged between 92 and 99 % with a LOD of 0.02 g/100 g of protein content.

8.2.2.4. Analysis of phenolic compounds

Total polyphenols were extracted from 0.2 g of lyophilized extracts in 10 mL of ethyl acetate after 30 min of sonication (in a water bath ultrasonicator, Ultrasonik 57H Ney). The polyphenols extract was filtered with anhydrous sodium sulphate (Sigma) and brought to dryness with a rotary evaporator (Laborato 4000, Heidolph). The residue was re-dissolved in 6 ml of milliQ water and aqueous phase was then submitted to a liquid-liquid extraction with diethyl ether (3 x 30 ml). The organic phases were evaporated at 40 ºC to dryness. Samples were derivatized by adding 250 µl of pyridine (Fluka, Switzerland), 250 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Fluka, Switzerland) and 50 µl of trimethylchlorosilane (TMCSI; Sigma Aldrich, Germany). Then, the samples were placed in a sand bath at 70º for 30 min. Derivatized samples were analysed by GC-MS (QP5000, Shimadzu, Maryland, USA) using a DB5 capillary column (0.25 µm film x 0.25 mm x 30 m). Helium was used as carrier gas, at a linear velocity of 35 cm/s. The sample (1 µL) was injected under splitless mode, at 250 ºC. The temperature program started at 80 ºC, and increased until 300 ºC at a rate of 8 ºC per min. The MS detector temperature was kept at 290 ºC. In a first step, GC-MS was operated in SCAN mode for identification of compounds by comparisons of their mass spectra with mass spectra in Wiley 27 and NIST 147 libraries (NIST 1999a,b and c), by mass fragmentograpphy, by comparison with standards as well as standard addition to the sample. No phenolic compounds were identified.

8.2.2.5. FTIR-ATR Analysis

Samples of lyophilized extracts were analysed by Fourier Transform Infrared Spectroscopy with attenuated total reflectance (FTIR-ATR) according to procedures described in chapter 3 - section 3.2.2.4.

8.2.2.6. ^{1}H NMR Analysis

For each lyophilized extract, 20 mg was suspended in 700 µL of D_2O and subsequently agitated in a vortex for 10 min. 650µL of the dissolved lyophilized extract was placed in 5mm NMR tubes (Aldrich528PP, 5 mm).

All spectra were acquired on a Bruker Avance 300 spectrometer with an operating frequency of 300.13 MHz. The acquisition of spectra was performed with a spinning rate of 20 Hz, a contact time of 4.75 s and with the pulse program ZG30. The recycle delay was 1 s and the length of the proton 90 pulses was 9.00 µs. About 56 scans were collected for each spectrum. A 0.3 Hz line broadening weighting function and baseline correction was applied. The identification of functional groups in the NMR spectra was based on their chemical shift (δ_H) relative to that of the water (4.7 ppm).

8.3. Results and Discussion

8.3.1. Rationale for selection of extracts

As mentioned above the selection of the four extracts was based mainly on their major potential in some of the tested biological activities. In Tables 8.1 and 8.2 the proximate composition as well as the main results for the studied biological activities (antioxidant, prebiotic and antidiabetic activities) in each different extract obtained from seaweeds and mushrooms is listed. No data of the extracts from *S. muticum* obtained by high hydrostatic pressure was considered in Table 8.1 because low values for antioxidant activity and absence of antidiabetic activity were observed for these extracts.

Table 8.1. Summary analysis of proximate composition as well as of antioxidant (ABTS, DPHH; $HO^{\cdot}$•, $O_2^{\cdot}$•), prebiotic and antidiabetic activities of seaweed extracts from *S. muticum*, *O. pinnatifida* and of *C. tomentosum*.

	Extraction Method	Extraction Yield	Proximate characterization				Antioxidant				Prebiotic		Antidiabetic
			Nitrogen	Sugars	Sulfated sugars	Total phenolic content	ABTS	DPPH	$HO^{\cdot}$•	$O_2^{\cdot}$•	La-5	BB12	α-GIA
S. muticum	HWE	(-)	(+)	(-)	(-)	(+)	(++)	(++)	(++)	(-)	(+)	(+)	(-)
	UAE	(-)	(+)	(-)	(-)	(++)	(++)	(++)	(+)	(-)	(++)	(+)	(-)
	EAE_Cell	(-)	(+)	(+)	(-)	(++)	(++)	(++)	(++)	(-)	(-)	(++)	(-)
	EAE_Visc	(-)	(+)	(-)	(-)	(++)	(+)	(+)	(++)	(-)	(++)	(+)	(-)
	EAE_Alc	(-)	(++)	(-)	(-)	(++)	(++)	(++)	(++)	(-)	(++)	(++)	(-)
	EAE_Flv	(-)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(-)	(++)	(+)	(-)
O. pinnatifida	HWE	(++)	(-)	(+)	(++)	(-)	(-)	(-)	(-)	(++)	(-)	(-)	(-)
	UAE	(+)	(-)	(+)	(++)	(-)	(-)	(-)	(-)	(++)	(-)	(-)	(-)
	EAE_Cell	(++)	(-)	(+)	(++)	(-)	(-)	(-)	(+)	(++)	(-)	(-)	(+)
	EAE_Visc	(++)	(-)	(+)	(++)	(-)	(-)	(-)	(+)	(+)	(+)	(++)	(+)
	EAE_Alc	(++)	(-)	(-)	(+)	(-)	(+)	(-)	(+)	(++)	(-)	(-)	(-)
	EAE_Flv	(++)	(+)	(+)	(++)	(-)	(+)	(-)	(++)	(++)	(-)	(-)	(-)
C. tomentosum	HWE	(+)	(+)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(+)
	UAE	(++)	(+)	(-)	(-)	(-)	(-)	(++)	(+)	(-)	(-)	(++)	(-)
	EAE_Cell	(++)	(+)	(+)	(-)	(-)	(-)	(++)	(-)	(-)	(++)	(++)	(+)
	EAE_Visc	(++)	(-)	(++)	(-)	(-)	(-)	(++)	(-)	(-)	(+)	(+)	(+)
	EAE_Alc	(++)	(-)	(-)	(-)	(-)	(+)	(+)	(++)	(-)	(+)	(+)	(++)
	EAE_Flv	(++)	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(++)

Extraction yield (%): 23 - 36 (-); 37 - 50 (+); 51 - 63 (++)
Nitrogen ($mg/g_{lyoph\ extract}$): 11 - 17 (-); 18 - 23 (+); 24 – 30 (++)
Sugars ($mg_{glucose\ equiv}/g_{lyoph\ extract}$): 30 - 71 (-); 72 - 113 (+); 114 - 158 (++)
Sulfated sugars ($\mu g_{Na2SO4\ acid\ equiv}/g_{lyoph\ extract}$): 6 - 24 (-); 25 - 40 (+); 41 – 60 (++)
Total phenolic content ($\mu g_{cathecol\ equiv}/g_{lyoph\ extract}$): 200 - 330 (-); 331 - 460 (+); 461 - 601 (++)
ABTS (% scavenging activity): 5 – 14 (-); 15 – 24 (+); 25 - 34 (++)
DPPH (% scavenging activity): 1 – 7 (-); 8 – 14 (+); 15 - 21 (++)
$OH^{\cdot}$• (% scavenging activity): 35 – 39 (-); 40-45 (+); 46 - 51 (++)
$O_2^{\cdot}$• (% scavenging activity): 1 – 9 (-); 10 – 17 (+); > 18 - 27(++)
La5 (Log cfu/mL):No increment of viable cells after 48h in comparison to 0h (-); No increment of viable cells after 48h in comparison to 0h but higher than values obtained with FOS (+);Similar or higher values of viable cells after 48h in comparison to 0h and higher than values obtained with FOS (++)
BB12 (Log cfu/mL): No increment of viable cells after 48h in comparison to 0h (-); No increment of viable cells after 48h in comparison to 0h but higher than values obtained with FOS (+);Similar or higher values of viable cells after 48h in comparison to 0h and higher than values obtained with FOS (++)
α-glucosidase inhibitory activity (% Inhibition): 29 - 36 (-); 37 - 42 (+); 43 - 49 (++)

Taking into consideration the results displayed in Table 8.1 selection fell on the enzymatic extracts of *S. muticum* obtained with Alcalase and of *O. pinnatifida* obtained with Viscozyme, both highlighted in the respective table. The enzymatic extract of *S. muticum* obtained with Alcalase was chosen based on its potential to increase the number of viable cells of *L. acidophilus* La-5 and *B. animalis* BB12 in comparison to the conventional prebiotic FOS and on the best results achieved in terms of antioxidant activity albeit its low potential to inhibit α-glucosidase as well as low extraction yield. *Sargassum muticum* is an invasive brown seaweed in Europe with high content of antioxidants such as carotenoids and phenols (Milledge et al., 2015) and therefore its use as a potential food ingredient would be an added value helping to control its invasive character. The selection of the enzymatic extract of *O. pinnatifida* obtained with Viscozyme was based on its potential in terms of antidiabetic and prebiotic activities and good extraction yield associated with good content of sulphated sugars. Extracts from the green seaweed *C. tomentosum* were not considered further due to their extremely high values of Na, around 200 $mg/g_{dry\ seaweed}$ (Chapter 3, Figure 3.1) which immediately limits its potential interest (from a nutritional perspective) to be used as a possible ingredient despite its interesting potential for antidiabetic activity in the extracts

obtained with Alcalase and Flavourzyme. Extracts from *Sargassum muticum* obtained from high hydrostatic pressure (HHP, Chapter 6) extraction were also not considered further because, unfortunately no antidiabetic activity was observed in these extracts.

Table 8.2. Summary analysis of proximate composition as well as of antioxidant (ABTS, $HO^{\bullet}$), prebiotic and antidiabetic activities of mushroom extracts from *Ph. nameko.*

	Extraction Method	Extraction Yield	Proximate characterization			Antioxidant		Prebiotic		Antidiabetic
			Nitrogen	Sugars	Phenolic content	ABTS	$HO^{\bullet}$	La-5	BB12	α-GIA
Ph. nameko	HWE	(+)	(+)	(+)	(-)	(+)	(+)	(++)	(++)	(+)
	UAE	(+)	(+)	(++)	(-)	(-)	(++)	(++)	(++)	(++)
	EAE_Cell	(++)	(-)	(++)	(-)	(-)	(-)	(++)	(++)	(++)
	EAE_Visc	(+)	(-)	(+)	(+)	(+)	(++)	(++)	(++)	(+)
	EAE_Alc	(-)	(++)	(-)	(+)	(++)	(+)	(++)	(+)	(-)
	EAE_Flv	(-)	(+)	(+)	(++)	(+)	(+)	(++)	(++)	(++)

Extraction yield (%): 54 - 61 (-); 62 - 69 (+); 70 - 77 (++)
Nitrogen ($mg/g_{lyoph\ extract}$): 40 - 43 (-); 44 - 47 (+); 48 – 52 (++)
Sugars ($mg_{glucose\ equiv}/g_{lyoph\ extract}$): 376 - 459 (-); 460 - 543 (+); 544 - 628 (++)
Total phenolic content ($\mu g_{cathecol\ equiv}/g_{lyoph\ extract}$): 144 - 156 (-); 157 - 169 (+); 170 - 182 (++)
ABTS (% scavenging activity): 37 – 40 (-); 41 – 43 (+); 44 - 47 (++)
$OH^{\bullet}$ (% scavenging activity): 51 – 53 (-); 54-56 (+); 57 - 60 (++)
La5 (Log cfu/mL):No increment of viable cells after 48h in comparison to 0h (-); No increment of viable cells after 48h in comparison to 0h but higher than values obtained with FOS (+);Similar or higher values of viable cells after 48h in comparison to 0h and higher than values obtained with FOS (++)
BB12 (Log cfu/mL): No increment of viable cells after 48h in comparison to 0h (-); No increment of viable cells after 48h in comparison to 0h but higher than values obtained with FOS (+);Similar or higher values of viable cells after 48h in comparison to 0h and higher than values obtained with FOS (++)
α-glucosidase inhibitory activity (% Inhibition): 14 - 25 (-); 26 - 37 (+); 38 - 50 (++)

Taking into account the results displayed in Table 8.2 it was selected the enzymatic extracts of *Ph. nameko* obtained with Cellulase and with Flavourzyme both highlighted in the respective table. The best potential in terms of antidiabetic and prebiotic activities of both extracts was the main reason for their selection. The selection of the enzymatic extract obtained with Flavourzyme over extract obtained with ultrasound assisted extraction (UAE) was mainly due to its potential of total antioxidant capacity (ABTS) and higher phenolic content despite its lower extraction yields.

8.3.2. Elemental inorganic and organic composition of seaweeds and mushroom enzymatic extracts

The elemental inorganic composition of the selected aqueous enzymatic extracts of *S. muticum* and *O. pinnatifida* are shown in Table 8.3. For the majority of the macro and microelements increments were observed in comparison to the concentrations found in the corresponding dry seaweeds. High content of ash in polysaccharides of marine origin namely in sulphated polysaccharides have been reported (Costa et al., 2012).

Table 8.3. Elemental inorganic and organic composition of enzymatic seaweeds extracts

			EA_*S. muticum* Alcalase		EA_*O. pinnatifida* Viscozyme	
			($mg/g_{lyophilized\ extract}$)	Ratio[1]	($mg/g_{lyophilized\ extract}$)	Ratio[1]
Inorganic	Macro elements	K	407±13	7.1	174±3	6.7
		Na	66±2	1.8	222±3	2.4
		Ca	2.31±0.06	0.3	8.34±0.04	1.5
		Mg	29.3±0.3	2.0	36.8±0.8	7.7
		P	11.6±0.5	5.1	6.30±0.09	3.6
	Micro elements	Zn	0.033±0.002	1.3	0.34±0.02	5.9
		B	0.319±0.004	3.0	0.28±0.01	2.2
		Mn	0.045±0.004	4.1	0.34±0.06	29.2
		Fe	0.17±0.02	0.9	1.8±0.1	4.9
		Al	<LOD	-	0.085±0.07	0.6
		Cu	0.065±0.002	14.4	0.026±0.001	5.3
		Ni	<LOD	-	0.20±0.01	-
		Pb	0.040±0.001	-	0.020±0.001	-
Organic		%N	2.5	-	1.3	-
		%C	17.1	-	16.8	-
		%H	2.5	-	3.5	-
		%S	0.7	-	1.7	-

[1]Ratio = Content in lyophilized extract/Content in dry seaweed; Values of organic elements are presented as average of triplicate samples.

It is evident from Table 8.3 that the inorganic macroelements were found at higher concentrations in both lyophilized marine seaweed extracts in comparison to respective mineral contents of dried seaweed counterparts reported in chapter 3. Despite the higher contents in minerals, concentration factors thereof, represented by the calculated ratio, differed between both macroelement and extract origin. The main constituent in both extracts was indeed K and it was also the macroelement with the highest ratio associated in the case of the *S. muticum* extract (ratio of 7.1, Table 8.3) and the second highest in the case of the *O. pinnatifida* extract (ratio of 6.7, Table 8.3). In the latter case the macroelement with the highest ratio associated was Mg (ratio of 7.7, Table 8.3), albeit its almost 5-fold lower content (compared to K content). Magnesium levels were in the same range of values in both extracts and represented the third major macroelement found in both extracts (after K and Na). Magnesium has been reported as an important mineral for cardiovascular function (Krishnaiah et al., 2008). The *S. muticum* extract also enabled a significant concentration of the P macroelement with a ratio of 5.1 (Table 8.3). Phosphorus is essential because it is part of the skeletal structure and teeth but also has other important functions such as the contribution to the control of the acid-base balance in the blood and in the carbohydrate metabolism it contributes to the intestinal absorption of glucose by the process of phosphorylation (Pérez et al., 2005).

Although the widespread increase in the content of the various macroelements, the ratio Na/K diminished in both extracts in comparison to the corresponding dry seaweeds. In the case of the *S. muticum* extract, the ratio Na/K diminished four-fold from 0.65 to 0.15 whereas the three-fold decrease for *O. pinnatifida* extracts was of larger amplitude, from 3.6 to 1.3. Extracts with low ratio of Na/K are good possibilities to be used as salt substitutes as already discussed in chapter 3.

The microelement content in both extracts varied; in general, the *O. pinnatifida* extract was much richer in microelement composition than the *S. muticum* counterpart, particularly in Fe, Zn and Mn, with contents 10-fold higher. High increment variability was observed for the microelements found in both extracts. Noteworthy, is the high increment of Cu and Mn in *S. muticum* and in *O. pinnatifida* extracts, respectively (Table 8.3) in comparison to the corresponding dried seaweeds content. Iron ratio in *S. muticum* extract indicates a slight decrease relatively to the dried seaweed content but its content increased 5 times in the *O. pinnatifida* extract (ratio of 4.9; Table 8.3) comparatively to the dried seaweed, and represents the major microelement found in this extract. Iron is an important mineral being a component of various enzymatic systems with a main function in the transport of oxygen from the lungs to the tissues: Iron deficiency is one of the most common nutritional disorders worldwide which can cause anaemia (Allen et al., 2006).

According to elemental inorganic composition of the two seaweeds extracts, two main observations can be made: 1) enzymatic aqueous extraction enables concentration of the majority of the macro and microelements, and 2) from a functional perspective, enzymatic extract of *S. muticum* obtained with Alcalase could be a good contributor to K, Mg as well as P (added value of extract given the concentration factor for P) recommended daily intakes (RDIs) whereas enzymatic extract of *O. pinnatifida* obtained with Viscozyme could be a good contributor not only of Fe (similarly to the dried seaweed) but also of K, Mg, Zn and Mn (added value) RDIs.

Concerning the organic elemental results shown in Table 8.3 for the seaweeds extracts, relatively similar compositions were observed with slightly higher content in N and C for the *S. muticum* enzymatic extract. This tendency correlates well with the higher nitrogen content found in this extract in comparison with the *O. pinnatifida* enzymatic extract (Chapter 5, Table 5.2). Higher content in H and S was in turn observed in *O. pinnatifida* enzymatic extract which is also in agreement with the higher content of polysaccharides, namely sulphated polysaccharides in this extract in comparison with the *S. muticum* enzymatic extract (Chapter 5, Table 5.2).

Similarly to the seaweeds extracts, increments of the majority of macro and microelements was also observed in both *Ph. nameko* enzymatic extracts in comparison to the corresponding dry mushroom (Table 8.4).

Table 8.4. Elemental inorganic and organic composition of enzymatic *Ph. nameko* extracts

			EA_*Ph. nameko* Flavourzyme		EA_*Ph. nameko* Cellulase	
			($mg/g_{lyophilized\ extract}$)	Ratio[1]	($mg/g_{lyophilized\ extract}$)	Ratio[1]
Inorganic	Macro elements	K	126±1	3.4	98.0±1	2.6
		Na	20±0.4	-	29.7±0.9	-
		Ca	0.6±0.1	2.6	0.24±0.02	1.1
		Mg	6.3±0.2	4.0	5.5±0.2	3.5
		P	25.1±0.6	2.3	22.4±0.4	2.1
	Micro elements	Zn	0.36±0.02	2.9	0.311±0.004	2.6
		B	0.043±0.001	1.7	0.041±0.002	1.7
		Mn	0.120±0.004	6.8	0.108±0.002	6.1
		Fe	0.34±0.03	1.2	0.28±0.01	1
		Al	<LOD	-	<LOD	-
		Cu	0.086±0.002	3.1	0.073±0.002	2.7
		Ni	<LOD	-	<LOD	-
		Pb	0.008±0.001	-	0.006±0.001	-
Organic		%N	5.3	-	4.4	-
		%C	37.6	-	36.0	-
		%H	9.0	-	8.5	-
		%S	0.6	-	0.8	-

[1]Ratio = Content in lyophilized extract/Content in dry mushroom; Values of organic elements are presented as average of triplicate samples.

Not much difference is observed among the inorganic elemental compositions of the two mushroom enzymatic extracts selected. It is evident from Table 8.4 that K and P are the main macroelements present in the two mushroom extracts. The contents of K are especially high in comparison to Na, an advantage from the nutritional point of view. Magnesium is the third major macroelement found in extracts and levels were similar in both mushroom extracts analysed. Calcium was not significantly present in the two mushroom extracts nevertheless the *Ph. nameko* Flavourzyme extract contained three-fold higher amounts than the *Ph. nameko* Cellulase extract. In comparison to the macroelement composition of the dried mushrooms (Chapter 3) significant increments in composition of between 2.1 and 4.0 were observed for the macroelements Mg, K and P (in descending order of magnitude) in both mushroom extracts. An important increment of 2.6 was also observed for Ca in the enzymatic extract obtained with Flavourzyme. Overall, both enzymatic extracts seem to be good sources of these macroelements.

In terms of microelements, Mn, Zn and Cu are among those with increments between 2.6 and 6.8. Iron and Zn are the most abundant elements among the trace minerals in the dry mushrooms and in the corresponding aqueous enzymatic extracts. As already mentioned Fe is known to be essential for cellular energy and metabolism (Jankowska et al., 2013) whereas Zn is also important because it is present in all organs, tissues, fluids, and secretions participating in all major biochemical pathways (Brown et al., 2004). Zinc is involved in the structure and function of over 300 enzymes, especially in superoxide dismutase (Zheng et al. 2014). Zinc deficiency can cause several problems namely adverse outcomes of pregnancy, sickle cell disease, metabolic syndrome and diabetes (Miao et al., 2013; Prasad, 2002; King, 2000).

Equally important but from a food safety perspective are the very low levels of Pb registered in both mushroom extracts – levels even lower than those reported in literature for different species of edible mushrooms (Mattila et al., 2001). When mushrooms are produced in substrates that contain these elements accumulation may occur and it is known that dietary excess of Pb may be detrimental to health. The organic elemental contents were similar in both *Ph. nameko* extracts as shown in Table 8.4, although a slightly higher content in N, C and H was indeed observed for the *Ph. nameko* extract obtained with Flavourzyme which could be related to the higher nitrogen content found in this extract. On the other hand, the enzymatic extract obtained with Cellulase was registered a higher content in polysaccharides (Chapter 7, Table 7.1).

8.3.3. Monosaccharides

The composition of monosaccharides, uronic acids and amino-monosaccharides in seaweeds extracts is displayed in Table 8.5. The most abundant in both seaweed extracts were galactose, mannose, glucuronic acid and glucosamine.

Table 8.5. Composition in monosaccharides, uronic acids and amino-monosaccharide in enzymatic extracts of seaweeds *S. muticum* and *O. pinnatifida*.

		*S. muticum*_Alcalase ($mg/g_{lyophyzed\ extract}$)	*O. pinnatifida*_Viscozyme ($mg/g_{lyophyzed\ extract}$)
Monosaccharides	DL-Glucose	<LOD	<LOD
	DL-Galactose	19.1±0.3	25.3±0.2
	DL-Mannose	7.8±0.2	11.4±0.2
	DL-Arabinose	0.10±0.01	0.16±0.01
	D-Xylose	3.23±0.02	4.8±0.1
	L-Rhamnose	0.27±0.01	0.52±0.01
	L-Fucose	4.3±0.1	5.60±0.09
Uronic acids	D-Glucuronic acid	17.4±0.3	27.3±0.2
	D-Galacturonic acid	1.07±0.01	1.50±0.02
Amino-mon.	D-glucosamine	7.9±0.1	12.7±0.4

Research on polysaccharides from marine seaweeds reveals water-soluble polysaccharides composed of a variety of sugars in variable molar ratios. Marine brown seaweeds are known to contain alginate, laminaran and sulphated polysaccharides known as fucoidans (Herrero et al., 2013). Fucoidans are made up of fucose, galactose, mannose, xylose, glucose, uronic acids, sulphate substituents, and acetyl groups which can also contain some protein components (Balboa et al., 2013). According to Dore et al. (2013), fucan sulphated polysaccharides extracted from the brown seaweed *Sargassum vulgare* were composed of fucose, galactose, xylose, glucuronic acid

and mannose whose proportion varied according to the fractions of the fucans extracted. The proportion of these monosaccharides found in the *S. muticum* extract obtained with Alcalase was of 0.22:1:0.17:0.91:0.41 (Table 8.5), which evidences the presence of fucoidans in the extract. Several studies on fucans from the *Sargassum* genus indicate that they are generally composed of glucuronic acid, mannose and galactose residues with partially sulphated-chains consisting of galactose, xylose and fucose (Dore et al., 2013). Aqueous extracts of *S. muticum* alginate-free solids presented variable relative proportions of uronic acids and fucose, galactose, xylose, manose and glucose (Balboa et al., 2013). It must be highlighted that fucoidans may differ considerably in composition, molecular mass and structure, depending on several factors such as species, geographic location, environmental conditions, harvest season, life-stage as well as extraction (Balboa et al., 2013). Some brown seaweed fucoidans have a backbone of 3-linked α-L-fucopyranose, whereas in other cases the backbone presents alternating 3- and 4-linked α-L-fucopyranose residues and sulphated galactofucans (Bilan & Usov, 2008), which are prominently found in various *Sargassum* species (Duarte et al., 2001). These are mainly built of (1→6)-β-D-galactose and/or (1→2)-β-D-mannose units with branching points formed by (1→3) and/or (1→4)-α-L-fucose, (1→4)-α-D-glucuronic acid, terminal β-D-xylose and sometimes (1→4)-α-D-glucose (Duarte et al., 2001).

The presence of glucosamine in appreciable amount (7.9 $mg/g_{lyophlized\ extract}$) in the *S. muticum* extract obtained with Alcalase suggests the presence of a proteoglycan-like material as those reported by García-Ríos et al. (2012) in *Sargassum filipendula*.

Higher contents of monosaccharides, uronic acids and amino-monosaccharide were observed in *O. pinnatifida* extract obtained with Viscozyme than in the *S. muticum* extract (Table 8.5) which is in agreement with the total sugars and sulphated sugars quantified in these extracts (Table 5.2, Chapter 5). The composition of monosaccharides of *O. pinnatifida* extract rich in galactose with smaller quantities of xylose is in accordance to agaran polysaccharides (Duarte et al., 2002). According to FTIR-ATR (Chapter 3), the red seaweed *O. pinnatifida* was considered an agar-like producer seaweed. Agarans are galactans biosynthesized by red seaweeds constituted by 3-linked β-D-galactose alternating with 4-linked α-L-galactose units presenting different degrees of cyclization of the α-L-galactose residues to give 3,6-anhydro-α-L-galactose (Canelón et al., 2014). In addition, agarans usually have a certain degree of substitution with sulphate ester groups, methyl ethers, pyruvate ketals, D-xylose and/or 4-O-methyl-L-galactose side chains, and different percentages of 3,6-anhydrogalactose (Usov, 2011). Higher content of galactose with variable proportions of xylose, mannose, fucose and glucose is reported by Canelón et al. (2014) for extracts of red seaweed *Laurencia* spp. obtained with water at room temperature and at 90 ºC. No data was found in the literature for red seaweed *O. pinnatifida*.

Table 8.6. Composition in monosaccharides, uronic acids and amino-monosaccharide in enzymatic extracts of mushroom *Ph. nameko*.

		*Ph. nameko*_Flavourzyme ($mg/g_{lyophyzed\ extract}$)	*Ph. nameko*_Cellulase ($mg/g_{lyophyzed\ extract}$)
Monosaccharides	DL-Glucose	28.3±0.4	31.1±0.3
	DL-Galactose	40.3±0.4	30.9±0.4
	DL-Mannose	16.2±0.2	17.9±0.3
	DL-Arabinose	0.49±0.01	0.46±0.01
	D-Xylose	0.29±0.01	0.21±0.01
	L-Rhamnose	<LOD	<LOD
	L-Fucose	<LOD	<LOD
Uronic acids	D-Glucuronic acid	<LOD	<LOD
	D-Galacturonic acid	<LOD	<LOD
Amino-mon.	D-glucosamine	3.7±0.1	4.5±0.1

The composition in monosaccharides and amino-monosaccharide in the two enzymatic extracts of mushroom *Ph. nameko* is displayed in Table 8.6. In general, the qualitative and quantitative profiles were quite different from those of the seaweed extracts presented in Table 8.5; monosaccharides were the major constituents found, with hexoses predominating over pentoses, associated contents were much higher and undetectable levels of uronic acids were also observed. The extract obtained with Flavourzyme was characterized by higher contents of monosaccharides with predominance of galactose (45%), glucose (32%) and mannose (18%). In comparison, the extract obtained with Cellulase possesses lower content of galactose (36%) which is in turn similar to glucose (36%), similar contents of mannose (21%) but a higher content of glucosamine (5.3%). Glucose, mannose and galactose were the most abundant monosaccharides found in crude polysaccharides and in their fractions of *Lentinus edodes*, *Grifola frondosa* and *Trametes versicolor*, with absence of uronic acids (Siu et al., 2014). According to Zheng et al. (2014) a zinc polysaccharide of *Ph. nameko* was composed of glucose, mannose, galactose and arabinose in a molar ratio of 33.0:4.5:4.3:1.0. A different molar ratio was in turn reported by Li et al. (2010) for a polysaccharide from *Ph. nameko* with hypolipidemic effect composed mainly of mannose, glucose, galactose, arabinose and xylose (1:8.4:13.6:29.6:6.2).

According to Wasser (2002) mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1→3), (1→6)-β-glucans and (1→3)-α-glucans but also as heteroglucans; in this case, side chains contain glucuronic acid, xylose, galactose, mannose, arabinose, or ribose as a main component or in different combinations. Since no glucuronic acid was detected in the two enzymatic extracts of *Ph. nameko*, it could indicate absence of heteroglucans. According to FTIR-ATR spectra (Chapter 3), the presence of β-glucans, α-glucans and glucan-protein complexes are among the main representative polysaccharides in the *Ph. nameko* mushroom.

Glucosamine was present in similar amounts (3.7-4.5 $mg/g_{lyophilized\ extract}$) in both enzymatic extracts of *Ph. nameko*. Glucosamine was also present in the crude polysaccharides and in their fractions of *L. edodes*, *G. frondosa* and *T.s versicolor* (Siu et al., 2014). Chitin, a polymer of N-acetyl- glucosamine alongside with β-glucans, is the primary structural material in mushrooms and has been shown to be of value as dietary fibre which can be hydrolysed to glucosamine (Sonawane et al., 2013; Nitschke et al., 2011).

8.3.4. Amino acids

The composition of free amino acids in seaweed and mushroom extracts is displayed in Table 8.7. Very low contents (1.4-2.7 $g/100g_{protein}$) and diversity of amino acids is observed in the seaweed extracts. Apparently the higher content in nitrogen content in the *S. muticum* extract obtained with Alcalase did not reflect in a higher content of free amino acids, probably in the form of peptides.

Table 8.7. Composition in free amino acids in enzymatic extracts of seaweeds *S. muticum* and *O. pinnatifda* and of mushroom *Ph. nameko.*

	*S. muticum*_Alcalase ($g/100g_{Protein}$)	*O. pinnatifida*_Viscozyme ($g/100g_{Protein}$)	*Ph. nameko*_Flavourzyme ($g/100g_{Protein}$)	*Ph. nameko*_Cellulase ($g/100g_{Protein}$)
Aspartic acid	0.24±0.07	2.0±0.2	1.26±0.09	2.4±0.1
Glutamic acid	0.7±0.2	0.7±0.1	-	1.02±0.09
Methionine	0.46±0.03	-	1.5±0.1	1.5±0.2
Serine	-*	-	0.21±0.01	0.9±0.1
Glutamine	-	-	-	0.25±0.06
Alanine	-	-	4.5±0.2	1.0±0.1
Leucine	-	-	0.25±0.02	-
Threonine	-	-	1.2±0.1	-
Valine	-	-	2.98±0.01	-

*Not detected above the limit of detection.

Higher contents and diversity of free amino acids is observed for both enzymatic extracts of *Ph. nameko*. The enzymatic action of Flavourzyme, a multi-enzyme complex with endo-protease and exo-peptidase, resulted in a higher nitrogen content (Chapter 7, Table 7.1) and higher content of free amino acids (11.9 $g/100g_{protein}$) than the Cellulase action ($7.1g/100g_{protein}$). Out of the nine different kinds of amino acids which constitute the nitrogen moiety of the *Ph. nameko* extract obtained with Flavourzyme, the major amino acids appeared to be alanine (38%), followed by valine (25%), methionine (13%), aspartic acid (11%) and threonine (10%). On the other hand, aspartic acid (34%), methionine (21%), glutamic acid (15%), threonine (14%) and serine (13%) were the major amino acids in the extract obtained with Cellulase treatment. Noticeably, the essential amino acids methione, valine, threonine and leucine make up 50% of the amino acid composition of the Flavourzyme *Ph. nameko* extract. Of particular interest is the fact that no aromatic amino acids, phenylalanine and tyrosine, were detected in either of the *Ph. nameko* extracts. Indeed, both phenylalanine and tyrosine are key intermediates in the synthesis of phenolic acids which may be responsible for the expression of antioxidant activity as observed in particular

for the *Ph. nameko* extract obtained with Flavourzyme. However no phenolic compounds were detected in both *Ph. nameko* extracts (section 8.2.2.4).

8.3.5. FTIR-ATR characterization

8.3.5.1. Seaweeds and their extracts

In Figure 8.1 the spectra of *S. muticum* and of its enzymatic extract obtained with Alcalase is displayed. High similarity between the two spectrums is visible with the majority of the bands present in *S. muticum* seaweed being also present in the enzymatic extract with Alcalase and practically only differences in absorption intensity are observable. Therefore some protein content, in agreement with previous results for nitrogen content (Chapter 5, Table 5.2) and free amino acids (Table 8.7), in the extract is expectable since N-H stretching vibrations at 3700-2900 cm^{-1} as well as from amide I and amide II at 1700-1420 cm^{-1} are present in both spectrum and could be related to proteins.

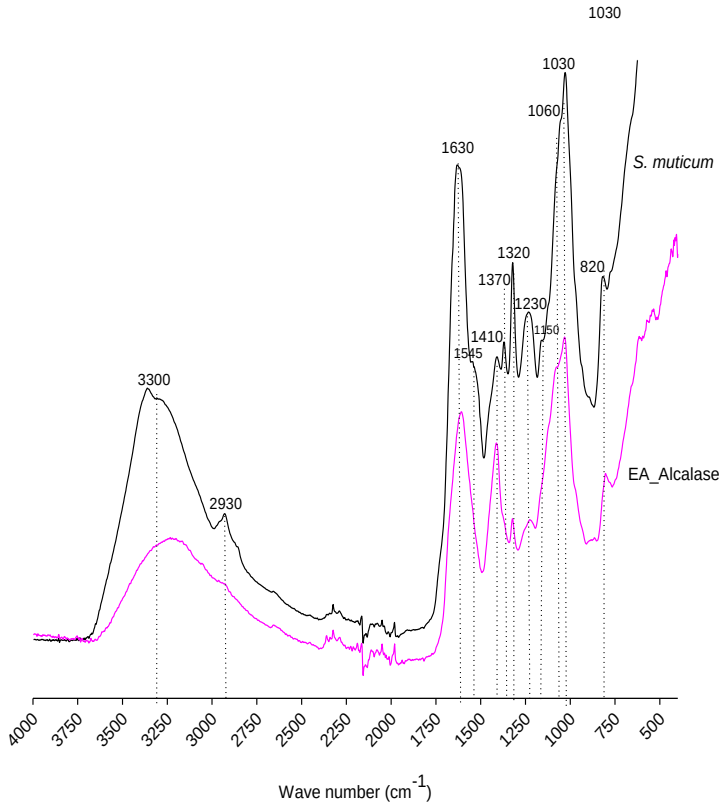

Figure 8.1. FTIR-ATR spectra of the brown edible seaweed *S. muticum* (—) and of its enzymatic extract obtained by Alcalase (—).

A broad band at 3280-3350 cm^{-1} and a weaker signal at 2870-2960 cm^{-1} could be assigned to O-H and C-H stretching vibrations but also to N-H stretching vibrations, respectively (Gómez-Ordóñez & Rupérez, 2011). The two characteristic absorptions, a band at around 1630 cm^{-1} (C-O asymmetric stretching vibration) and a band of 1410 cm^{-1} (C-O symmetric stretching vibration)

(Zheng et al., 2014), indicates the presence of carboxyl groups in both *S. muticum* seaweed and in its extract. In the *S. muticum* Alcalase extract spectrum there is a noteworthy intensity increment of the 1410 cm^{-1} band in comparison to other bands in the extract spectrum. This could indicate higher presence of protein or amino acids in the extract due to the role of the endopeptidase Alcalase. This enzyme was in fact responsible for a significantly higher nitrogen content ($p<0.05$) in the *S. muticum* extract (Chapter 5, section 5.3.2).

A focus on the 700-1400 cm^{-1} region is related with the seaweeds polysaccharides namely for carragenans and agar in red seaweeds and alginates and fucoidans in brown seaweeds as specified by several authors (Chapter 3, Table 3.3). Alginate, is the polysaccharide which has been found in brown seaweeds such as *S. muticum,* known to be a linear copolymer of β-D-mannuronic acid and α-L-guluronic acid (1-4)-linked residues arranged in heteropolymeric and/or homopolymeric blocks (Pereira et al., 2003). The presence of these acids can be evidenced especially by the bands around 1030 and 1060 cm^{-1} assigned to guluronic acid and at 1320 cm^{-1} assigned to mannuronic acid, all present both in *S. muticum* seaweed and in its extract obtained by Alcalase. In terms of the broad band around 1220-1260 cm^{-1} in the FTIR-ATR spectrum, assigned to the presence of sulphate ester groups (S=O) which is a characteristic component in fucoidan and other sulphated polysaccharides that can be found in some brown seaweeds (Pereira et al., 2013; Gómez-Ordónez & Rupérez, 2011), it is particularly observable in the *S. muticum* seaweed spectrum and less in its extract obtained by Alcalase (Figure 8.1). These results are in agreement with the monosaccharides composition observed previously for *S. muticum* extract which evidences the presence of fucoidans (Table 8.5).

In terms of *O. pinnatifida* seaweed and its enzymatic extract obtained by Viscozyme, a multi-enzyme complex of carbohydrases (Arabanase, Cellulase, β-Glucanase, Hemicellulase and Xylanase), the two spectra present some qualitative differences in the region 1100 to 1600 cm^{-1}. In the extract spectrum the bands at 1535 and 1212 cm^{-1} almost disappeared whereas the band at 1150 cm^{-1} increased in comparison to the *O. pinnatifida* seaweed spectrum (Figure 8.2).

The absorbance bands at 1222 and 1150 cm^{-1} are characteristic of less sulphated polysaccharides such as agar. Strong absorption at 1220-1260 cm^{-1} have been report by Yu et al. (2012) for agaran-type polysaccharide isolated from *Grateloupia filicina* and according to the data presented in chapter 3, O*. pinnatifida* was considered a red seaweed agar-like producer. The increment in the band 1150 cm^{-1} can be related with possible role of the multi-enzyme complex of carbohydrases on matrix polysaccharides (agar) and on cellulose, xylan and manan fibrils of the complex composite cell walls of red seaweeds (Domozych, 2011). The extract of O*. pinnatifida* was characterized by the highest content of sulphated sugars (Table 5.2, chapter 5). The absorbance band at 930 cm^{-1} present in both spectra has been assigned to the presence of 3,6-anhydro-D-galactose found in carragenan and agar polysaccharides (Pereira, 2006). These results are in accordance to the monosaccharides composition observed previously for *O. pinnatifida* extract which evidences the presence of agaran polysaccharides (Table 8.5).

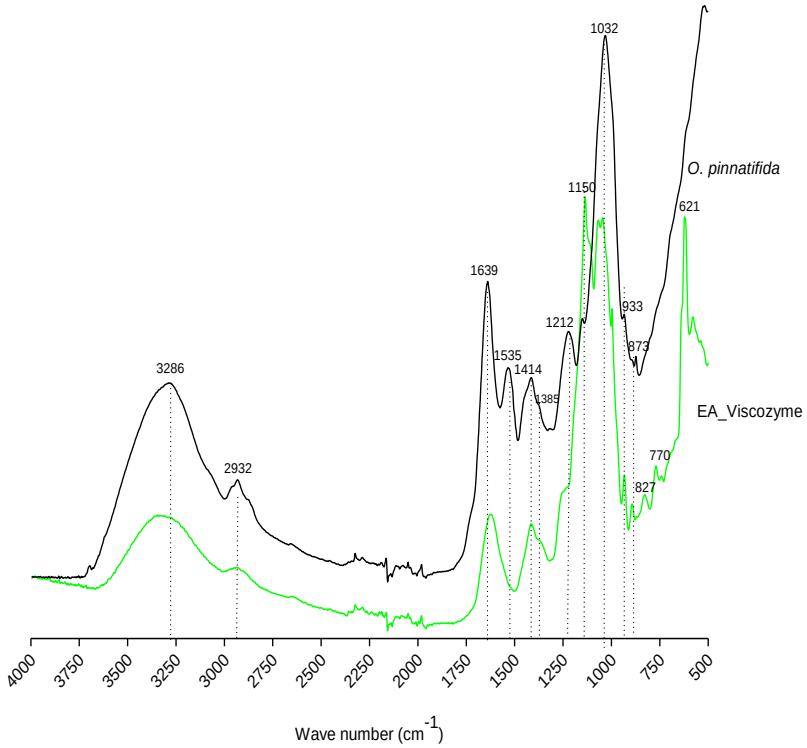

Figure 8.2. FTIR-ATR spectra of the red edible seaweed *O. pinnatifida* (—) and of its enzymatic extract obtained by Viscozyme (—).

Since the region 1700-1420 cm^{-1} has been attributed to amide I and amide II which in turn could be related to protein, the high reduction of absorbance band at 1535 cm^{-1} probably is due to some loss of nitrogen content during the extraction process. This correlates well with the fact that enzymatic extracts of *O. pinnatifida* were characterized by low nitrogen content (Chapter 5, Table 5.2) and low content of free amino acids (Table 8.7).

8.3.5.2 *Pholiota nameko* and their extracts

FTIR-ATR spectra of mushroom *Ph. nameko* and of its enzymatic extracts (EA) obtained by treatment with Cellulase and Flavourzyme with are depicted in Figure 8.3. Some differences, in terms of qualitative and absorbance intensity, are observed between spectra of the mushroom and respective extracts. However, the four specific regions described in chapter 4, section 4.3.4. (4000-1800 cm^{-1}, 1800-1500 cm^{-1}, 1500-750 cm^{-1} and 950-750 cm^{-1}) are observable in the three spectra:
i) Considering the first region between 4000-1800 cm^{-1}, the prominent band centred around 3300 cm^{-1} may be assigned to O-H stretching vibrations of glyosidic structures. According to Klaus et al. (2015) characteristic N-H vibration at 3400 cm^{-1} could be overlapped by O-H stretch vibration at 3000–3500 cm^{-1} of inter- and intra-hydrogen bonds that are present in polysaccharides. The bands

around 2900-2880 cm^{-1}, in turn assigned to CH_2 and CH_3 stretching of fatty acids from the cell wall (Zheng et al., 2014; Zhao et al., 2006 a,b), are more defined in the mushroom spectrum than in the respective extracts spectra, in particular for the 2900-2880 cm^{-1}. This fact could result from loss of fatty acids as consequence of the extraction process.

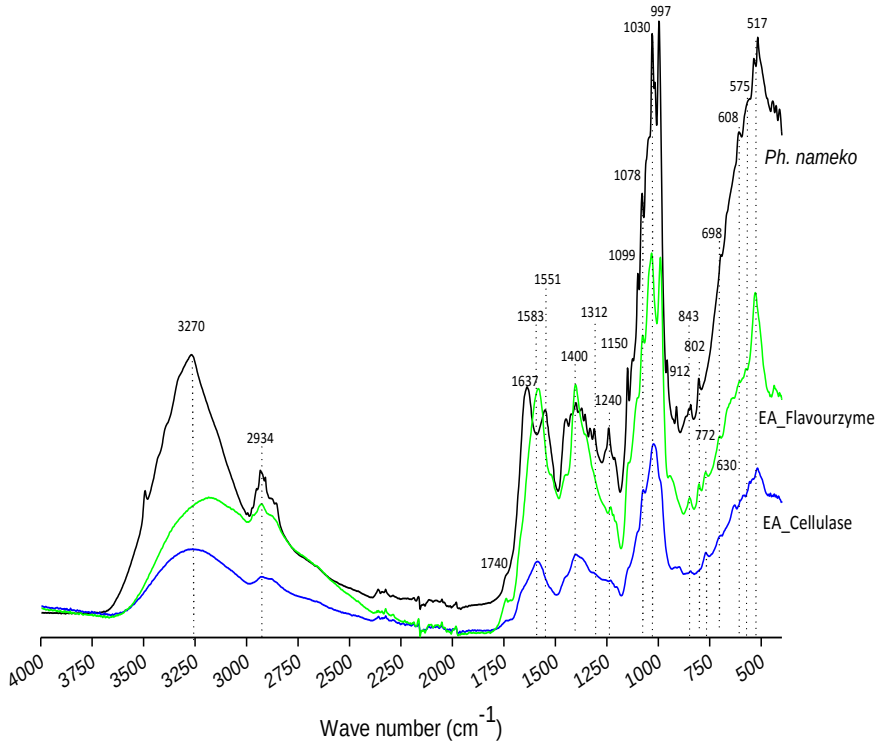

Figure 8.3. FTIR-ATR spectra of the cultivated mushroom *Ph. nameko* (—) and of its enzymatic extracts obtained by Flavourzyme (—) and Cellulase (—).

ii) In the region between 1800 and 1500 cm^{-1}, two major bands around 1600 and 1500 cm^{-1} assigned to amide I and amide II of proteins (Klaus et al., 2015; Zhao et al., 2006 a,b) are visible in mushroom spectra but not in the respective extracts. In the enzymatic extracts only the band around 1580 cm^{-1} is observable being much more pronounced for the Flavourzyme extract spectrum than for its Cellulase counterpart; such observation may be related to the role of the Flavourzyme, with its endo-proteases and exo-peptidases, on *Ph. nameko* proteins which effectively resulted in a higher nitrogen content (Chapter 7, Table 7.1) and a higher free amino acids content comparatively to the Cellulase extract (Table 8.7). In addition an absorbance band at 1740 cm^{-1} corresponding to carbonyl stretching vibration of alkyl-esters (Zhao et al., 2006 a,b) is present in the *Ph. nameko* and its Flavourzyme extract spectra but not in its Cellulase extract spectrum.

iii) In the region 1500-750 cm^{-1} which is associated with vibrations of proteins, lipids and polysaccharides, absorption bands at 1400 and 1240 cm^{-1} are visible both in the mushroom and in

its extracts spectra. These bands have been associated to protein structures (Gonzaga et al., 2005) which could be due to the presence of glucan-protein complex and role of the Flavourzyme. However according to Zheng et al. (2104), the absorption band around 1400 cm^{-1} could also suggest the occurrence of uronic acids (O-C=O bending) which were, however, not detected in both enzymatic extracts (Table 8.6). Other authors in turn indicate that absorption bands between 1410 and 1310 cm^{-1} could be assigned to OH groups of phenolic compounds (Klaus et al., 2015). Indeed, the *Ph. nameko* Flavourzyme extract was characterized by higher contents of nitrogen and total phenolic compounds but lower content of sugars in comparison to the Cellulase extract counterpart (Chapter 7, Table 7.1). However no phenolic compounds were detected by GC-MS (section 8.2.2.4).

The presence of polysaccharides in both enzymatic extracts of *Ph. nameko* could be confirmed by the presence of bands around 1150, 1070 and 1040 cm^{-1} in figure 8.3. The band 1040 cm^{-1} has been assigned to C-O stretching, 1070 cm^{-1} to the anomeric C_1H group vibration and 1150 cm^{-1} to C-O-C stretching of glycosidic structures (Klaus et al., 2015; Gonzaga et al., 2005). According to Zheng et al. (2014), the strong characteristic absorption at 1200–1000 cm^{-1} is ascribed to sugar ring vibrations overlapping with stretching vibrations of C-OH side groups and the C-O-C glycosidic bonds vibration. Wang et al. (2014) reported that each polysaccharide fraction from *Phellinus nigricans* mycelia had a specific band from 1200 to 1000 cm^{-1}. Higher band intensity at 1200–1000 cm^{-1} is observable in the *Ph. nameko* Flavourzyme extract than in its Cellulase extract. This could be related to the role of Cellulase on degradation of *Ph. nameko* polysaccharides; in fact, the *Ph. nameko* Cellulase extract contained higher sugar content than the *Ph. nameko* Flavourzyme extract but a slightly lower content of monosaccharides and amino-monosaccharide (Table 8.6).

iv) In the 950-750 cm^{-1} region which has been associated with identification of anomeric configuration of polysaccharides, in particular the 890 cm^{-1} assigned to β-glycosides and 860-810 cm^{-1} to α-glycosides (Klaus et al., 2015; Zheng et al., 2014; Zhao et al., 2006a,b), those bands are clearly present in the spectrum of the *Ph. nameko* Flavourzyme extract but not as intense in the spectrum of the Cellulase extract in accordance with the bands pattern in the region 1200–1000 cm^{-1} described above.

8.3.6. ^{1}H NMR spectroscopy

The ^{1}H NMR spectra of the seaweeds and *Ph. nameko* enzymatic extracts are quite similar (Figures 8.4 and 8.5, respectively). The spectra exhibit some distinct peaks overlaying much broader bands, as would be expected from the NMR spectra of complex mixtures of organic compounds (Santos et al., 2012).

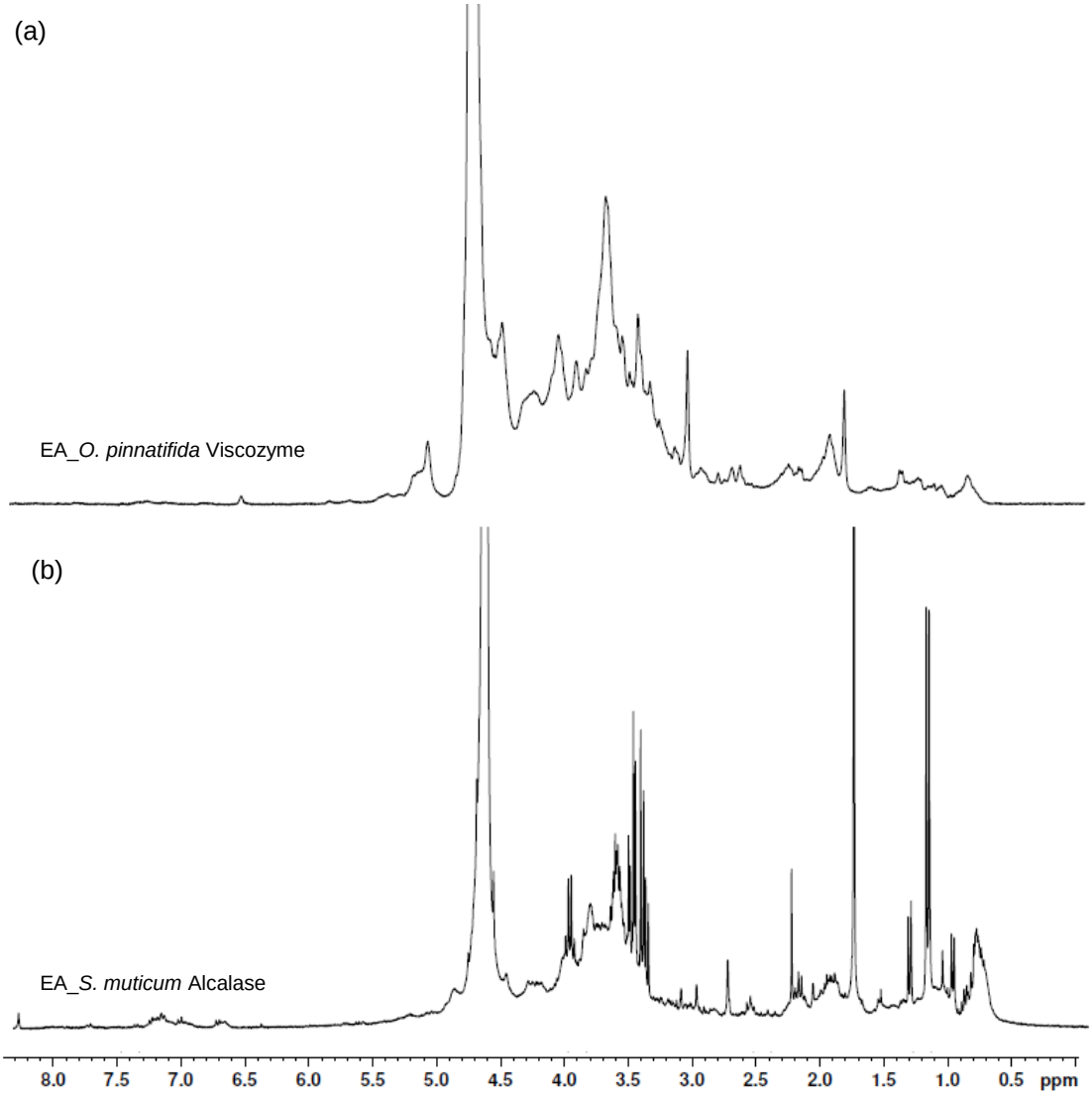

Figure 8.4. ^{1}H NMR spectra of enzymatic extracts of (a) *Osmundea pinnatifida* obtained by Viscozyme and (b) *Sargassum muticum* obtained by Alcalase. The peak at 4.7 ppm indicates the water signal.

Despite the large variety of overlapping resonances, each ^{1}H NMR spectrum was investigated on the basis of the chemical shift assignments described in the literature for organic compounds (Lopes et al., 2015; Santos et al., 2012; Clarke & Haselden, 2008). In accordance, four main regions of chemical shifts were considered in each spectrum:

1) δ_H = 0.6–1.8 ppm: aliphatic protons, H–C; -CH>-CH_2>-CH_3;

2) δ_H = 1.8–3.2 ppm: protons bound to carbon atoms in alpha position to unsaturated groups in allylic (H-C_α-C=), carbonyl or imino (H-C_α-C=O or H- C_α-C=N) groups, and protons in secondary and tertiary amines (H-C=NR_2 and NR_3);

3) δ_H = 3.2–4.1 ppm: aliphatic protons on carbon atoms singly bound to oxygen atoms (H–C–O: H-C-O-CO-R>H-C-OH or H-C-O-C) in alcohols, polyols, ethers and esters;

4) δ_H = 6.5–8.5 ppm (aromatic protons).

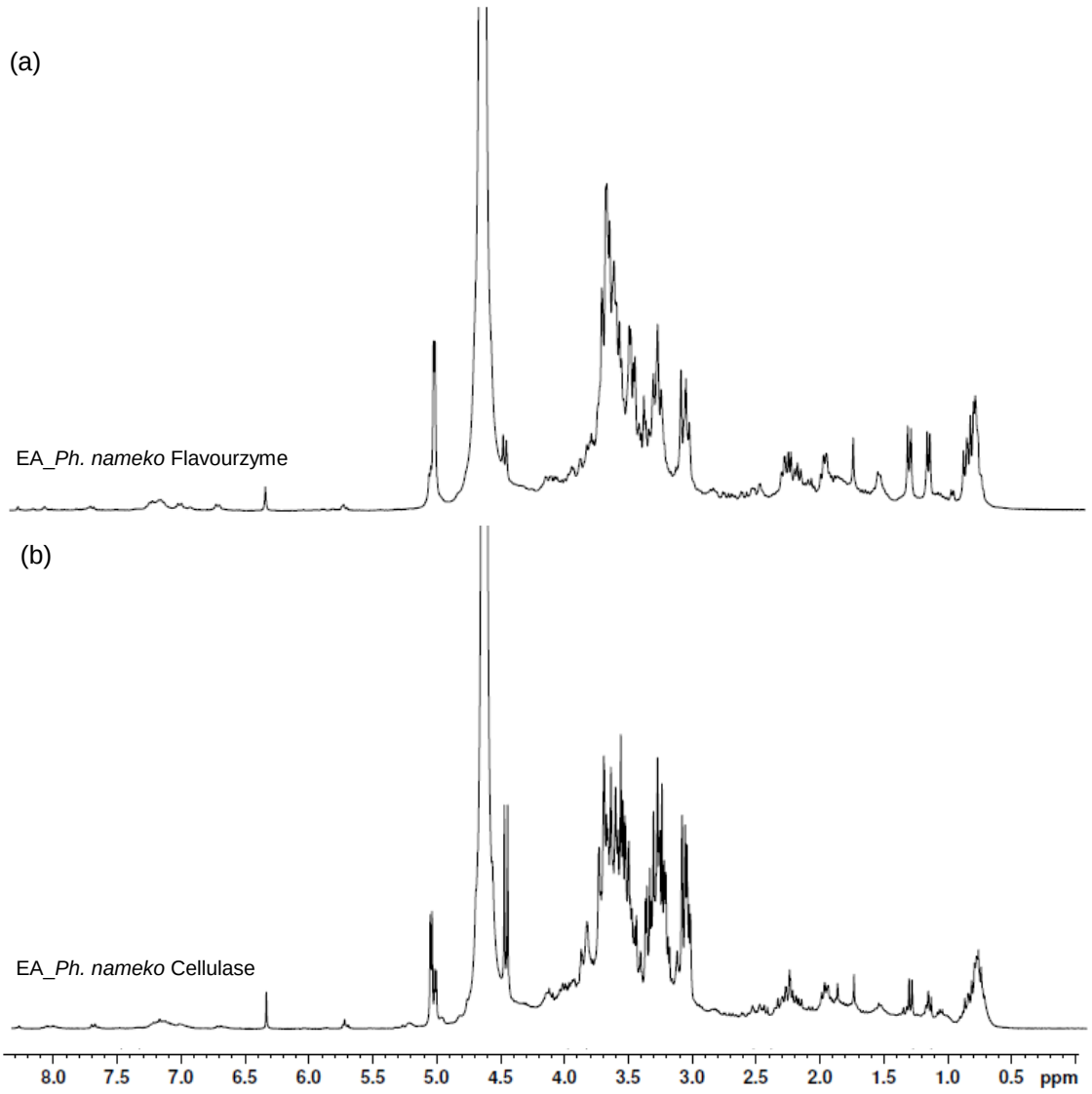

Figure 8.5. ^{1}H NMR spectra of enzymatic extracts from *Pholiota nameko* obtained by Flavourzyme (a) and Cellulase (b). The peak at 4.7 ppm indicates the water signal.

For a further understanding of the ^{1}H NMR data, a quantitative integration of each spectral region was performed in order to assess the abundance of each of the different types of protons in the different extracts and depicted in Figure 8.6. A fifth region (δ_H = 4.1–6.0 ppm) corresponding to anomeric protons of glycosidic structures (Gonzaga et al., 2005) was also considered but not integrated due to the wide and intense peak at 4.7 ppm associated with the water signal.

In accordance to the spectra, the relative abundance of each type of protons is, in general, relatively similar for the different extracts but some points are worthy of being highlighted. In terms of seaweeds extracts the higher percentages of protons belong to the group of aliphatic H-C directly bound to an oxygen atom (H-C-O) probably due to the presence of nonaromatic ring structures such as sugars (Clark & Haselden, 2008); higher values (67%) are observed for the

enzymatic extract of *O. pinnatifida* obtained with Viscozyme than for the enzymatic extract of *S. muticum* (43%) obtained with Alcalase. These values are in accordance with tendencies previously discussed for the FTIR-ATR spectra and sugars: the content of total sugars and of sulphated sugars in the enzymatic extract of *O. pinnatifida* obtained with Viscozyme is 2.3 and 8 times higher than in the enzymatic extracts of *S. muticum* obtained with Alcalase, respectively (Table 5.2, Chapter 5). According to Bubb (2003), ^{1}H spectra of carbohydrates do contain some well-resolved signals, including those of anomeric protons (δ_H = 4.4–5.5 ppm), acetyl (~δ_H = 2.0–2.1 ppm) and methyl (~δ_H = 1.2 ppm) groups as well as other protons that are influenced by specific functionality including amino groups, phosphorylation, sulphation, glycosylation and acetylation or lack of functionality as in deoxy-sugars. Signals due to polyols have been described by Tanniou et al. (2015) who studied the biochemical composition of *S. muticum* populations by ^{1}H HRMAS (High resolution magic angle spinning) NMR and assigned chemical shifts to polyols (mannitol) at δ_H = 3.5–4.0 ppm.

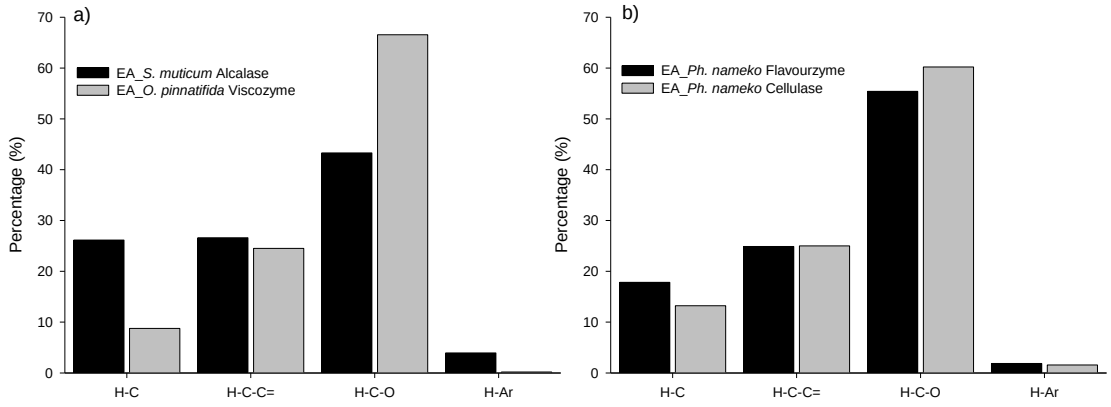

Figure 8.6. Relative abundance of each type of protons, estimated as the partial integrals of the spectra reported in Figures 8.4 and 8.5 for the enzymatic extracts of seaweeds (a) and of *Ph. nameko* (b): H-C: purely alkylic hydrogen atoms; H-C-C=: hydrogen atoms in alpha position to C=C or C=O groups; H-C-O: aliphatic C-H directly bound to an oxygen atom; H-Ar: aromatic hydrogen atoms.

Not many differences are observed for protons in alpha position to unsaturated groups in allylic (H-C_α-C=), carbonyl or imino (H-C_α-C=O or H- C_α-C=N) groups for both seaweed extracts but the slightly higher value for the enzymatic extract of *S. muticum* (27%) could be related to Alcalase activity which lead to 2.4 times higher content of nitrogen compounds in comparison to the enzymatic extract of *O. pinnatifida* obtained with Viscozyme (Table 5.2, Chapter 5). According to Gonzaga et al. (2005) a chemical shift at 2.78 ppm could be assigned to protein groups, which is visible in the spectrum of the *S. muticum* enzymatic extract but not in the corresponding *O. pinnatifida* enzymatic extract (Figure 8.4). Proteins have been described to be associated to cell wall polysaccharides being part of the structure of the seaweed cell walls as mentioned in the previous section (Robic et al., 2009, 2008).

In terms of purely alkylic hydrogen atoms (H-C; 26%) and of aromatic hydrogen atoms (H-Ar; 4%) the enzymatic extract of *S. muticum* is richer (26 and 4%, respectively) in these atoms than the enzymatic extract of *O. pinnatifida* (8.9 and 0.2%, respectively). Signals at δ_H = 7.2–7.3 ppm and δ_H = 7.6–7.8 ppm (Figure 8.4) are consistent with the presence of aromatic units containing both electron-donor (e.g. phenolic groups) and electron-acceptor (e.g. carbonyl and carboxyl) substituents. The content of phenolic contents in aqueous extracts has been shown to be low; albeit contents of 290 and 123 $\mu g_{cathecol\ equiv}/_{glyoph\ extract}$ in the enzymatic extracts of *S. muticum* and *O. pinnatifida*, respectively (Table 5.2, Chapter 5). However phenolic compounds were not detected by GC-MS analysis (section 8.2.2.4). Signals due to the presence of unsaturated fatty acids have been described by Tanniou et al. (2015) in *S. muticum* at δ_H = 1.0-1.5 ppm.

In the anomeric spectral region (δ_H = 4.1–6.0 ppm) different patterns are observed for both seaweeds extracts (Figure 8.4). In the case of the enzymatic extract of *O. pinnatifida* signals in the region δ_H = 4.2–4.5 and ~δ_H = 5.1 ppm could be assigned to α and β reducing end units whereas no particular signals are well resolved in this region for the enzymatic extract of *S. muticum.* According to Barros et al. (2013) the signal from an anomeric proton at δ_H = 5.13 was assigned to 3,6-α-L-anhydrogalactose while the signal at δ_H = 4.56 was attributed to β-D-galactose for polysaccharides of a red seaweed *Gracilaria caudata*. No literature references were found for ^{1}H NMR spectra of red seaweed *O. pinnatifida*, hence no associated comparison can be established. According to Llanes et al. (1997) the absence of signals in the regions δ_H = 5.0–5.3 ppm and δ_H = 4.7–4.9 ppm expected for anomeric protons of α and β reducing end units liberated (D-anomeric protons of mannuronosyl and L-guluronosyl residues from hydrolysis of sodium alginate from *Sargassum* sp.) are indication of limited hydrolysis of the *Sargassum* alginate or resulted from overlapping resonance with water signal at 4.7 ppm.

For both enzymatic *Ph. nameko* extracts more similarity is observed for the designated 4 groups of protons (Figure 8.5). Only the *Ph. nameko* extract obtained with Cellulase revealed slightly higher values for the H-C-O group which is probably related with the slightly higher content of sugars in this extract in comparison to that obtained with Flavourzyme (Chapter 7, Table 7.1). In addition, slightly higher content of purely alkylic hydrogen atoms (H-C) is observed for the *Ph. nameko* enzymatic extract obtained with Flavourzyme. Signals assigned to terminal methyl hydrogens H_3C–C ($\delta_H \approx$ 0.9 ppm) and to polymethylene chains $(CH_2)_n$ ($\delta_H \approx$ 1.3 ppm) are seen in both the spectra. No differences are observed for the other two groups of protons namely for the group of aromatic protons (H-Ar) and for the group of hydrogen atoms in alpha position to unsaturated groups in allylic (H-C_α-C=), carbonyl or imino (H-C_α-C=O or H- C_α-C=N) groups.

In the anomeric spectral region, a doublet at $\delta_H \approx$ 4.5 ppm and at ~ $\delta_H \approx$ 5.0 ppm particularly intense in the spectrum of the *Ph. nameko* enzymatic extract obtained with Cellulase, could be assigned to β and α configurations, respectively. These data are in agreement with FTIR-ATR analysis which assigned the presence of α- and β-glycosides and to data reported by Gonzaga et

al. (2005) for isolated polysaccharides from *Agaricus blazei* mushroom. No data for *Ph. nameko* was found in the literature.

8.4. Conclusions

An effective characterization of the four selected enzymatic extracts prepared by different enzymatic treatments of the seaweeds *O. pinnatifida* and *S. muticum* and the mushroom *Ph. nameko* was explored in this chapter. Once the important possibilities offered by these extracts were described, the composition in organic and inorganic elements, monosaccharides and amino acids was duly discussed and correlated whenever possible with results presented in previous chapters. Structure analysis of extracts was also achieved by FTIR-ATR and ^{1}H NMR.

This deeper characterization was of great importance since it enabled a more effective portrait of each selected extract, confirming results achieved in previous chapters on the one hand, and on the other adding new information to justify some of the biological behaviours observed and discussed in the previous studies.

In general, enzymatic extracts enabled the recovery of important compounds with nutritional or biological significance. Analysis of the elemental inorganic composition of the seaweeds and mushroom extracts showed that enzymatic aqueous extraction enabled an important concentration of almost all macro and microelements in comparison to the dry foods (seaweeds or mushroom), and in some cases nutritional value (an extract containing at least 15% of the mineral RDI value) was much enhanced, in particular for K and P in the *S. muticum* enzymatic extract; for K, Mg, Zn and Mn in the *O. pinnatifida* extract and for Mg, K and P in both *Ph. nameko* extracts.

Overall, the higher contents of monosaccharides, uronic acids and glucosamine which were observed in the *O. pinnatifida* extract obtained with Viscozyme in comparison to the *S. muticum* extract were in agreement with the total sugars and sulphated sugars previously quantified in these extracts as were further well correlated with the structural analysis obtained from the FTIR-ATR and ^{1}H NMR spectra. Results underlined the importance of such characterization since the two seaweeds revealed a variety of sugars in variable molar ratios. In the case of the mushroom extracts, equally a valuable source of water-soluble polysaccharides, qualitative and quantitative profiles contrasted with those of seaweeds; monosaccharides were the major constituents found at higher contents and no uronic acids were detected.

The nitrogen moiety found in these extracts, expressed as amino acids was not particularly expressive in the case of seaweeds extracts from both qualitative and quantitative standpoints yet significance improved for mushroom extracts. As expected the *Ph. nameko* extract obtained with Flavourzyme was characterized by a 1.7 higher total amount of amino acids in comparison to the Cellulase counterpart and interestingly half that amount was composed of important essential amino acids.

Structural analysis corroborated well previous results and explained well some of the differences in composition observed between the two seaweeds extracts or between the two *Ph. nameko* enzymatic extracts.

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Chapter 9

In vitro assessment of biological properties of selected seaweeds and mushrooms extracts

9.1. Introduction

As described in the previous chapter 8, two seaweeds extracts, namely, enzymatic extracts of *S. muticum* obtained with Alcalase and of *O. pinnatifida* obtained with Viscozyme, and two mushroom extracts, namely, enzymatic extracts of *Ph. nameko* obtained with Cellulase or with Flavourzyme, were selected mainly due to their potential biological properties. To be further explored within the functional food perspective there is a need to confirm and consolidate the biological potential of the selected extracts.

Prebiotics are defined as substrates that may improve the host health by selectively stimulating the growth and/or metabolic activity of one or a limited number of beneficial bacteria in the colon (Roberfroid et al., 2010). The potential prebiotic effect of the selected extracts has been evaluated by comparison with FOS, the gold standard in comparison studies, and using pure cultures as described in chapters 5 and 7. Nevertheless, the human gastrointestinal tract represents a complex ecosystem where the available nutrients will influence the growth of the gut microbiota (Roberfroid et al., 2010). Hence, to assess the possible importance that colonic catabolism of these extracts may have on human gut microbiota, *in vitro* batch culture fermentation experiments conducted with faecal inoculum from healthy volunteers, are in order to observe changes in the main bacterial groups present in the microbiota (Eid et al., 2014; Sánchez-Patán et al., 2012). The human colon is considered the most metabolically active site in the human body with live microorganism's cells that can reach to 10^{12}-10^{13} over 1000 species (O´Sullivan et al., 2010; Roberfroid et al., 2010). Faecal batch cultures, pH-controlled, enable to assess the fermentability of various substrates in the intestinal lumen, simulating the conditions in the human distal colon (Bergillos-Meca et al., 2015). A growing body of evidence suggests that the gut microbiota impacts a wide range of host metabolic pathways, barrier function and immune modulatory function influencing the prevention and risk of a wide range of diseases, including inflammatory bowel disease, diarrhea and colorectal cancer. Much of this impact is mediated through diet and the consumption of specific health-related foods, justifying the constant need to modulate diet or identify compounds that can positively modify the gut microbiota (Gibson et al., 2010).

Research has been focused on 'prebiotics', and in particular the ability of certain types of dietary fiber, especially indigestible oligosaccharides, to stimulate the growth of and/or activity of beneficial gut bacteria such as bifidobacteria and lactobacilli while retarding the development of *Clostridium histolyticum*, leading to a concomitant positive effect on colonic health (Gibson et al., 2010; Aida et al., 2009). Better understanding on the benefits of prebiotics has urged a need for search and development of new and alternative sources of prebiotics. According to Zaporohets et al. (2014), the prebiotic activity of extracts or of polysaccharides from marine seaweeds, combined with a broad spectrum of biological properties, evidences great potential for their use as functional nutrition ingredients enabling modulation of intestinal microbiota and of GIT inflammations as well as normalization of the immune system. Therefore, one of the main objectives in this chapter was to evaluate the potential of the digested (closely simulating physiological conditions) seaweed and

mushroom extracts on gut microbial ecology. To my knowledge there are no studies regarding the impact of water-based enzyme-assisted extracts for *S. muticum*, *O. pinnatifida* or *Ph. nameko* on gut microbial ecology. Besides the confirmation of the prebiotic potential further assessment of antihypertensive modulatory activity was also envisaged in this chapter in order to confirm and consolidate the biological potential of the selected extracts, for their application as functional food and bioactive ingredient sources.

9.2. Material and methods

9.2.1. Selected seaweeds and mushrooms extracts

As described in the introduction section, the seaweed extracts selected for further biological assessment were the enzymatic extracts of *S. muticum* obtained with Alcalase and of *O. pinnatifida* obtained with Viscozyme. In terms of mushroom, the enzymatic extracts of *Ph. nameko* obtained with Cellulase and with Flavourzyme were the selected choices.

9.2.2. Biological properties

9.2.2.1. *In vitro* fermentation by human gut microbiota

Samples of the selected extracts were submitted to three consecutive steps: 1) Simulated gastrointestinal digestion, 2) Faecal batch-culture fermentation and, 3) Bacterial enumeration using fluorescence *in situ* hybridization (FISH).

9.2.2.1.1. Simulated gastrointestinal digestion

To simulate the digestion of the selected seaweed and mushroom extracts through the gastrointestinal tract and therefore evaluate the main effects of the digested extracts on human microbiota, samples were treated according to Mills et al. (2008), with slight alterations. Water (25 mL) was added to 10 g of lyophilized extract, and the mixture was stomached (Seward, UK) for 5 min using 200 paddle-beats per min. The extract solution was then mixed with α-amylase (3.33 mg) in $CaCl_2$ (0.001 M, pH 7.0; 1.04 mL) and incubated at 37 $^{\circ}$C for 30 min and at 130 rpm in a shaker. Afterwards, the pH was decreased to 2.0 with 6 M HCl and pepsin (P 7000, Sigma; 0.45 g) dissolved in HCl (0.1 M; 4.16 ml) was added. The sample was incubated at 37 ºC for 2 h and at 130 rpm in a shaker. After this period, the pH was increased to 7 with 6 M NaOH, and pancreatin (P 8096, Sigma; 0.93 g) and bile (B 8631, Sigma; 0.58 g) in $NaHCO_3$ (0.5 M; 20.8ml) were added. The extract solution was then incubated at 37^{o}C and at 45 rpm for 3 h and afterwards transferred to 1 kDa molecular weight cut-off regenerated cellulose dialysis tubing (Spectra/Por® 6, Spectrum Europe, Netherlands) and dialysed against NaCl 0.01 M at 5 $^{\circ}$C, to remove low molecular mass

digestion products. After 15h the NaCl dialysis fluid was changed and dialysis continued for an additional 2h. Afterwards the digested samples were frozen at -80 ºC and lyophilized in a freeze dryer (Armfield SB4 model, Ringwood, UK). All chemicals were purchased from Sigma (St Louis, USA).

9.2.2.1.2. Faecal batch-culture fermentation

Three independent fermentation experiments were carried out. Faecal samples were obtained fresh at the premises of the Department from 3 apparently healthy adult volunteers who ingested a normal diet, had not ingested any antibiotics for at least 6 months and were not regular consumers of pre or probiotics. Samples were collected into sterile vials and kept in an anaerobic cabinet and used within 30 min of collection. A 1/10 (w/w) dilution in phosphate buffer saline (PBS) was prepared and homogenised using a stomacher (Serward, Worthing, UK) for 2 min at 460 paddle-beats per min.

Sterile stirred batch culture fermentation vessels (50 mL working volume) were set up and aseptically filled with 45 mL sterile, pre-reduced, basal medium [peptone water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L, $MgSO_4$.7H2O 0.01 g/L, $CaCl_2.6H_2O$ 0.01 g/L, $NaHCO_3$ 2 g/L, Tween 80 2 mL/L (BDH, Poole, UK), Hemin 0.05 g/L, vitamin K1 10 µL/L, L-cysteine-HCl 0.5 g/L, bile salts 0.5 g/L, pH 7.0)] and gassed overnight with O_2-free N_2 (15 mL/min) with constant agitation. All media and chemicals were purchased from Oxoid (Basingstoke, UK) and Sigma (St Louis, USA). The temperature was kept at 37 ºC and pH was controlled between 6.7 and 6.9 using an automated pH controller (Fermac 260, Electrolab, Tewkesbury, UK), which added acid or alkali (0.25 M HCl and 0.25 M NaOH) in order to mimic conditions that resemble the distal region of the human large intestine (Sánchez-Patán et al., 2012).

Six stirred pH-controlled batch fermenters were run in parallel. Four vessels were aseptically added with each different digested extract (1% w/v) and the other two vessels were used as controls. To the positive control vessel it was added 1% (w/v) of FOS (95% oligofructose, 0.5 kDa dextran with 25% α-1,2 branching, 1 kDa dextran with 32% α-1,2 branching; *Orafti®P95*, Oreye, Belgium). To the negative control vessel no source of carbon was added. The experiment was performed in triplicate, using one faecal sample given by a different donor for each run of six batch fermenters. Each vessel, with 45 mL sterile medium and digested extract, was inoculated with 5 mL of fresh faecal slurry (1/10 w/w). The batch cultures were run under anaerobic conditions for a period of 24h, during which 5 mL samples were collected from each vessel at 0, 6, 12 and 24h for FISH and analysis of lactic acid and short chain fatty acids (SCFA). For this latter analysis, samples were stored at -70 ºC until required.

In order to assess the effect of addition of 2 and 3% of digested extracts of *O. pinnatifida* obtained with Viscozyme and of *Ph. nameko* obtained with Flavourzyme, faecal batch-culture

fermentations were repeated, in triplicate, under similar conditions but with lower volumes (10 mL) and for a period of 12h.

9.2.2.1.3. Bacterial enumeration using FISH

To assess differences in bacterial composition, FISH was used with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA based on the method described by Daims et al. (2005). A total of 6 different probes commercially synthesized and 5'-labelled with the fluorescent dye (Sigma Aldrich, St Louis, USA) were used in addition to an overall stain with 4,6-diamidino-2-phenylindole (DAPI), which measures all cells by staining DNA (Harmsen et al., 2000a,b). The used probes, their specificity and diagnostic regions of 16S rRNA as well as hybridization and washing conditions are displayed in Table 9.1.

Samples (375 µL) obtained from each vessel and sampling time, were fixed for a minimum of 4 h (4 ºC) in 1125 µL 4% (w/v) paraformaldehyde. Fixed cells were centrifuged at 13,000 g for 5 min and washed twice in 1 mL filtered sterilized PBS. The washed cells were re-suspended in 150 µL filtered PBS and stored in 150 µl ethanol (99%) at -20 ºC until further processing. Samples were then diluted in a suitable volume of PBS in order to obtain countable fluorescent cells in each field of view and 20 µl of the above solution was added to each well of a 6 well PTFE/poly-L-lysine coated slide (Tekdon Inc., Myakka City, USA). The samples were dried for 15 min in a drying chamber (46 ºC).

To permeabilize cells for use with probes Bif164 and Lab 158 (Table 9.1), samples were treated with 20 µL of lysozyme at room temperature for 15 min before being washed briefly in water. Slides were dehydrated, using an alcohol series (50, 80 and 96% (v/v) ethanol) for 3 min in each solution. Slides were returned in the drying oven for 2 min to evaporate excess ethanol before adding the hybridization mixture to each well [1 mL consisting in 180 µL of 5M NaCl, 20 µL of 1M Tris/HCl (pH 8.0), 799 µL of ddH_2O, 1 µL of 10% sodium dodecyl sulphate and 100 µL of probe (50 ng/µL)]. For probes EUB338 I-II-III, the hybridization mixture contained formamide [1mL consisting in 180 µL of 5M NaCl, 20 µL of 1M Tris/HCl (pH 8.0), 350 µL formamide, 449 µL of ddH_2O, 1 µL of 10% sodium dodecyl sulphate and 100 µL of probe (50 ng/µL)]. Hybridization occurred for 4 h in a microarray hybridization incubator (Grant-Boekel, Cambridge, UK). After hybridization, slides were washed in 40 mL washing buffer for 15 min. They were then dipped in cold water for a few seconds and dried with compressed air. Five microlitres of polyvinyl alcohol mounting medium with 1,4-diazabicyclo(2,2,2)octane (DABCO) was added onto each well and a cover slip was placed on each slide (20 mm, thickness No 1, VWR, Lutterworth, UK). Slides were examined by epifluorescence microscopy (Eclipse 400, Nikon, Surrey, UK) using the Fluor 100 lens. For each well, 15 fields with a maximum of 300 positive cells were counted.

Table 9.1. 16S rRNA oligonucleotide probes and hybridization conditions used in FISH analysis.

Probe name	Specificity	Sequence (5' - 3')	Hybridization pre-treatment	Formamide (%) in hybridization buffer	Temperature (ºC)		References
					Hybridization	Washing	
Bac 303	Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae*	CCAATGTGGGGGACCTT	None	0	46	48	Manz et al. 1996
Chis 150	Most of the *Clostridium histolyticum* group (Clostridium clusters I and II)	TTATGCGGTATTAATCTYCCTTT	None	0	50	50	Franks et. al 1998
Erec 482	Most of the *Clostridium* coccoides/*Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)	GCTTCTTAGTCARGTACCG	None	0	50	50	Franks et al. 1998
Lab 158	*Lactobacillus* spp./*Enterococcus* spp.	GGTATTAGCAYCTGTTTCCA	Lysosyme	0	50	50	Harmsen et al. 1999
Bif 164	*Bifidobacterium* spp.	CATCCGGCATTACCACCC	Lysosyme	0	50	50	Langendijk et al. 1995
EUB338-I-II-III	Members of the domain Bacteria	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	None	35	46	48	Daims et al. 1999

9.2.2.1.4. Lactic acid and SCFA analysis

Samples were collected from each batch culture at each sampling point (0, 6, 12 and 24h) and frozen at -70 ºC until required.

Samples were assessed for lactic acid and SCFA (acetic, propionic, butyric, isobutyric and isovaleric acids) using an HPLC apparatus from Merck LaChrom (Fullerton CA, USA), in a single run, based on calibration curves previously prepared with appropriate chromatographic standards; an Aminex HPX-87X cation exchange column from BioRad (Richmond CA, USA) was used for separation; the eluant was pumped at 0.8 mL/min, and consisted of 13 mM H_2SO_4 (Merck); and detection was by UV absorbance at 220 nm.

Prior to analysis, samples were defrosted, centrifuged (14000 rpm for 10 min at 4 ºC) and filtered through a 0.22-µm membrane filter (Millipore, USA) to remove all particulate matter.

9.2.2.2. Anti-hypertensive activity

The assay of angiotensin-I converting enzyme (ACE) was based on method developed by Sentrandreu & Toldrá (2006) with some modifications. To each microtiter-plate well (Nunc, Denmark), 160 µL of fluorescent substrate Abz-Gly-Phe(NO_2)-Pro (Bachem Feinchemikalien, Germany) and 40 µL of each extract, with different concentrations, were added. The enzyme reaction was initiated by the addition of 2 mU of ACE (peptidil-dipeptidase A, EC 3.4.15.1 Sigma Chemical, St. Louis, MO, EUA), dissolved in glicerol (50%) and prepared in buffer solution Tris-HCl (150 mM) with 0,1 mM de $ZnCl_2$, pH 8.3, that are immediately mixed and incubated at 37 ºC. The generated fluorescence was measured after 30 min by a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany). Excitation and emission wavelengths were 350 and 420 nm, respectively. The software used was Fluostar Control versión 1.32 R2.

9.2.3. Statistical Analysis

A paired Student's *t* test was used to test for significant differences in the bacterial group populations between extracts and controls as well as for time *in vitro* fermentation experiments by human gut microbiota.

9.3. Results and discussion

9.3.1. Modulation of intestinal microbiota by seaweed and mushroom digested extracts.

Assessment of the prebiotic potential of potential bioactive compounds or extracts by *in vitro* fermentation with human faecal microbiota provides a cost-effective and rapid alternative to assess the fermentation and modulation capacity of different substrates at laboratory scale on a

comparative basis (Gullon et al., 2014). For the three volunteers, changes in the different bacterial populations and accumulation of lactic acid and SCFA (acetic, propionic, butyric acids) during the experimental time course (0, 6, 12 and 24h) of the *in vitro* fermentation of the digested seaweed and mushroom extracts at 1% (w/v) were followed. For comparative purposes, similar experimental strategy was used with the well-established prebiotic FOS (positive control) as well as for faecal fermentation medium without carbon source present (negative control) (Figure 9.1). All data of bacterial populations (Log cell/mL) are expressed as average of three replicas (donors) plus standard deviation, justifying the high variability reported in several cases.

It is important to note that before performance of the *in vitro* fermentations care was taken to submit each extract to simulated gastrointestinal digestion because resistance to gastric acidity and hydrolysis by mammalian enzymes are limiting factors that have to be assured in order to enable the substrate to reach the colon and be fermented by intestinal microbiota, meeting the pre-requisite for a prebiotic effect or gut modulation effect. According to Gibson et al. (2010) any dietary material that is non-digestible and enters the large intestine is a candidate prebiotic. The few studies that have evaluated the prebiotic potential of seaweed polysaccharides using *in vitro* fermentation (laminarin and low molecular weight polysaccharides from agar and alginate) did not promote previous gastrointestinal digestion, hindering analysis of true effectiveness of intact compounds (Ramnani et al., 2012; Devillé et al., 2007).

FISH was used to monitor the modifications among populations of selected bacterial species caused by the different digested *S. muticum*, *O. pinnatifida* and *Ph. nameko* enzymatic extracts added at 1%, on a comparative basis. Depending on the bacterial group different effects were observed. Both enzymatic extracts of *Ph. nameko* and that of seaweed *O. pinnatifida* obtained with Viscozyme lead to overall increases in *Bifidobacterium* populations as compared to the negative control between 6 and 24h of fermentation, confirming a stimulatory effect (Figure 9.1a). Highest shift was observed for medium containing *Ph. nameko* extract obtained with Flavourzyme raising bifidobacterial counts from 8.06±0.66 Log cell/mL at 0h to 8.49±0.06 Log cell/mL at 24h (p=0.391) in comparison to the negative control at 24h (p=0.021). Evidence for a potential prebiotic effect for pure culture of *B. animalis* BB12 was observed for undigested *Ph. nameko* enzymatic extracts, except for the extract obtained with Alcalase (Chapter 7, section 7.3.3). The positive control FOS, did however induce a higher increase in bifidobacterial numbers after 24h of fermentation; from 8.04±0.47 at 0h to 8.98±0.13 at 24h (p=0.017); for example a two-fold higher increase was observed for FOS (0.94 log) when compared to *P. nameko* extract obtained with Flavourzyme (0.43).

Surprisingly, no significant lactobacilli populations changes were observed over the 24h in comparison to the negative control for any of the four extracts tested at 1% except for FOS (Figure 9.1b). These results do contrast with those reported and discussed in previous studies obtained with pure cultures of *L. acidophilus* La-5; recall the significantly higher values (p<0.05) of viable cell numbers observed for the majority of culture media enriched with seaweed water-based extracts (Chapter 5, section 5.3.4) and with *Ph. nameko* extracts (Chapter 7, section 7.3.3) after 24h of

incubation in comparison to growth in media with glucose or FOS. Nevertheless, Ramnani et al. (2012) also reported absence of effect on lactobacilli populations by low molecular weight polysaccharides from agar and alginate seaweeds.

The stimulation of growth of and/or activity of beneficial gut bacteria such as *Bifidobacterium* by the digested extract of *Ph. nameko* obtained with Flavourzyme associated with decrease in numbers of cells of *Clostridium histolyticum* after 24h in comparison to the negative control (Figure 9.1e) can be considered a concomitant positive effect on colonic health (Gibson et al., 2010; Aida et al., 2009). Furthermore, it must also be highlighted that the positive FOS promoted an increase in numbers of cells of *Clostridium histolyticum* after 12 and 24h in comparison to the negative control (Figure 9.1.e) whereas the opposite was observed in particular for the extracts of *O. pinnatifida*, *S. muticum* and *Ph. nameko* obtained with Flavourzyme. Although there is a clear difference in trend effect some authors have mentioned that an increase in *C. histolyticum* numbers may be consequence of culture conditions rather than a specific effect mediated by the tested prebiotic compounds (Bergillos-Meca et al., 2015). Nonetheless, these same authors reported such increase in *C. histolyticum* numbers for both positive control (FOS) and for tested probiotic/prebiotic conditions which is not the case presented herein. The digested extract of *S. muticum* obtained with Alcalase seems to be the less promising of the tested extracts considering the absence of a positive shift for both the *Bifidobacterium* and lactobacilli groups and their positive influence on the *Clostridium* groups at 6 and 12h.

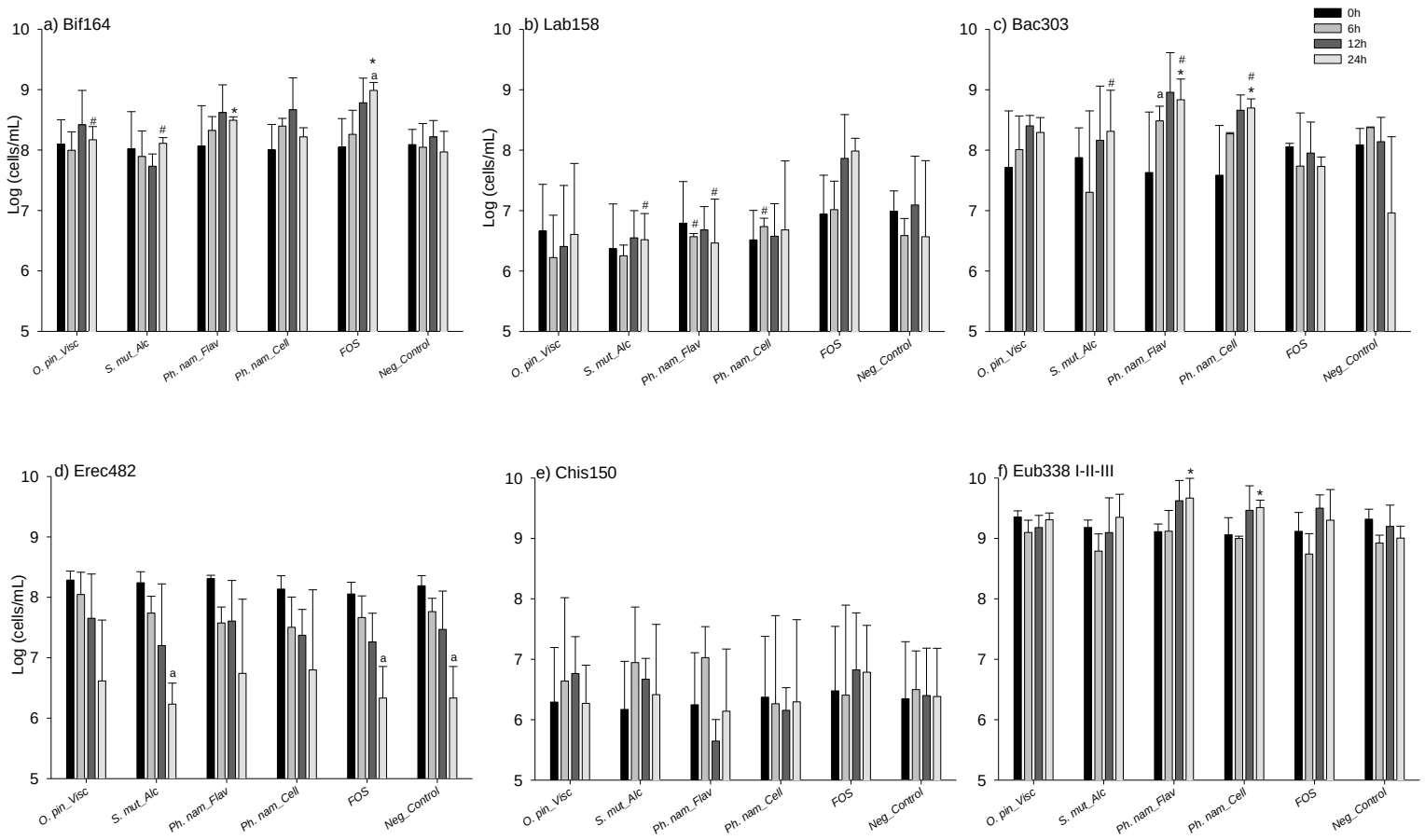

Figure 9.1. Bacterial populations (a) *Bifidobacterium* spp.; b) *Lactobacillus* spp./*Enterococcus* spp.; c) Bacteroides group; d) *C. cocoides*/*E. rectale* group; e) *C. histolyticum* group; f) Total bacteria analysed by FISH in batch cultures containing 1% (w/v) of digested extracts of seaweeds *O. pinnatifida* obtained by Viscozyme (O.pin_Visc) and *S. muticum* obtained by Alcalase (S.mut_Alc) and of mushroom *Ph. nameko* obtained by Flavourzyme (Ph.nam_Flav) and by Cellulase (Ph.nam_Cell) and the respective controls. Error bars indicate SD of the replicates involving 3 adult donors. $^{a}p<0.05$; significantly different compared to 0h within the same substrate. $^{*}p<0.05$; significantly different compared to negative control. $^{\#}p<0.05$; significantly different compared to FOS, positive control.

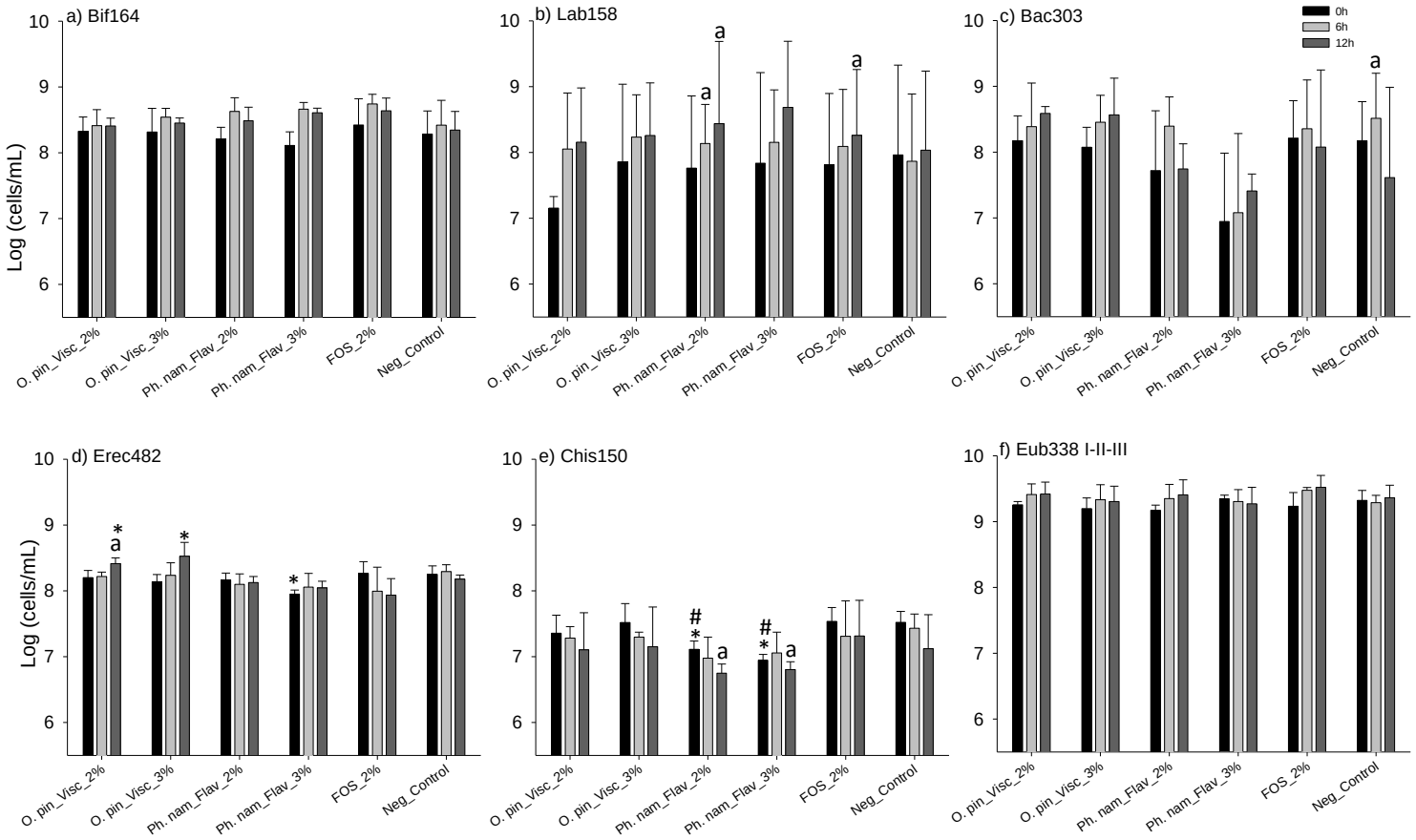

Figure 9.2. Bacterial populations (a) *Bifidobacterium* spp.; b) *Lactobacillus* spp./*Enterococcus* spp.; c) Bacteroides group; d) *C. cocoides*/*E. rectale* group; e) *C. histolyticum* group; f) Total bacteria analysed by FISH in batch cultures containing 2 and 3% (w/v) of digested extracts of seaweed *O. pinnatifida* obtained by Viscozyme (O.pin_Visc) and of mushroom *Ph. nameko* obtained by Flavourzyme (Ph.nam_Flav) and the respective controls. Error bars indicate SD of the replicates involving 3 adult donors. $^{a}p<0.05$; significantly different compared to 0h within the same substrate. $^{*}p<0.05$; significantly different compared to negative control. $^{\#}p<0.05$; significantly different compared to 2% FOS, positive control.

The four digested extracts at 1% lead to similar increases in numbers of total bacteria after 24h fermentation in comparison to the negative control, which actually reported a slight reduction in numbers by 24h (Figure 9.1f). Furthermore, the four digested extracts at 1% all lead to a decrease in numbers of the *C. cocoides/Eubacterium rectale* group (Figure 9.1d), which is a major anaerobic population in the human gut, similarly to the negative and positive controls. Statistical significant decreases (p<0.05) were observed for *S. muticum* extract alongside with both negative and positive controls for *C. cocoides*/*E. rectal*e group.

Bacteroides/*Prevotella* population showed an increase over the 24h of fermentation for all the four digested extracts, in particular for both extracts of *Ph. nameko* in comparison to the negative control or to the positive FOS which revealed no significant shift in numbers over the whole 24h fermentation period (Figure 9.1c). Although *Bacteroides*/*Prevotella* populations increase with the addition of the digested extracts to the medium it is important to correlate shift in population with the production of short chain fatty acids. *Bacteroides* and *Prevotella* genera are organisms capable of using a very wide range of substrates and are major producers of propionate. As may be seen form data listed in Table 9.2 and discussed further down in this chapter propionic acid production is significant by 24h fermentation, and of the same order of magnitude, for the positive control FOS and for both the *Ph. nameko* extracts, yet branched-chain SCFA are reduced, which is concurrent with a decrease in protein fermentation and of positive influence as far as *Bacteroides* modulation is concerned. It is known that these genera vary greatly with the nature of the diet and while studies have revealed increased proportions of *Bacteroides* in vegetarians (Matijasic et al., 2014) or upon ingestion of resistant starch type 4 (Martinez et al., 2010), others have detected no alterations in *Bacteroides* upon ingestion of formula diet containing FOS and pea fibre (Benus et al., 2010) or upon blueberry drink consumption (Vendrame et al., 2011) , or have shown reduction in the *Bacteroides* population as a consequence of ingestion of a mixture of galactooligosaccharides by overweight adults (Vulevic et al., 2013).

In order to observe if increasing concentrations of the digested enzymatic seaweeds and mushroom extracts would have higher impact on gut microbiota modulation, similar *in vitro* fermentations were repeated with 2 and 3% (w/v) of extracts of *O. pinnatifida* obtained with Viscozyme and of *Ph. nameko* obtained with Flavourzyme. The selection of these two extracts was based on the best prebiotic potential selectivity effect demonstrated within each group of extracts, seaweeds and mushroom. The respective results are displayed in Figure 9.2. Interesting results were obtained for *Bifidobacterium, Lactobacillus* and *Clostridium* groups (Figures 9.2a, 9.2b and 9.2.e), respectively: i) an increase in the concentration of the digested extracts did not bring about a higher impact on *Bifidobacterium* abundance and increases in population numbers were similar between digested extracts and the positive control FOS in comparison to the negative control which registered no alteration over 12h fermentation; ii) abundance in the lactobacilli group was significantly increased in comparison to the digested extracts at 1% fermentation (Figure 9.1b) where no significant increases had been observed for the extracts; iii) higher increases in lactobacilli populations, although not statistically significant (p>0.05), were observed between 0 and

6h for both concentrations of *Ph. nameko* extracts than with FOS at 2% and at 12h of fermentation similar numbers of cells were observed for both *Ph. nameko* extracts and FOS 2% and these were higher than those obtained with the negative control; iv) although the experiments with 2 and 3% *Ph. nameko* extract started with the lowest level of *C. histolyticum* in the faecal inocula these extracts brought about the only statistical significant decreases of *C. histolyticum* (p=0.0003 for 2% and p=0.028 for 3%, respectively) in comparison to 0 h and in comparison to FOS (p>0.05) and negative control (p>0.05). The fact that numbers of *C. histolyticum* continued to diminish with higher percentages of *Ph. nameko* Flavourzyme extracts (in comparison to 1% extract) confirms their selectivity properties and prebiotic potential. At higher percentages cross-feeding may become more predominant and selectivity could in fact be lost, yet this is not the case since *C. histolyticum* showed no proliferation and actually decreased in numbers. Another positive effect for these extracts was observed for the number of *Bacteroides* (Figure 9.2c) which did not increase in comparison to the negative control.

9.3.2 Lactic acid and SCFA production

Lactic acid and SCFA, the main products arising from the microbial fermentation of carbohydrates, can provide energy to the colonic epithelium, modulate cholesterol and lipid metabolism, suppress pathogenic intestinal bacteria and modulate the immune system (Salazar et al., 2009). Furthermore, they act as electron sinks of anaerobic respiration in the gut and decrease the intestinal pH, thus enhancing the bioavailability of minerals such as calcium and magnesium (Gullon et al., 2014). The concentration of acetic, propionic, butyric, iso-butyric and iso-valeric acids as well as lactic acid produced during 24h fermentation of the different extracts added at 1% is shown in Table 9.2. Significant differences were found between donors with respect to the levels of the different SCFA, in particular with acetic and butyric acids. In some cases the SCFA were found in only one donor, particularly for propionic, butyric and branched-chain fatty acids.

As expected, SCFA production in the negative control was the lowest (14.3 mM by 24h) in comparison with the media containing either the seaweed or the mushroom extracts. The lack of a carbohydrate may ascribe SCFA production in the negative control to protein degradation by putrefactive bacteria (Gullon et al., 2014). The total SCFA concentrations achieved were highest for medium containing the positive control FOS (75.1 mM by 24h), followed by medium added with the *Ph. nameko* extracts (50.9 and 50.5 mM by 24h for *Ph. nameko* Flavourzyme extract and *Ph. nameko* Cellulase extract, respectively) and lastly by media added with *O. pinnatifida* Viscozyme extract (26.7 mM) and *S. muticum* Alcalase extract (20.0 mM by 24h). Such global trends correlate well with the observed modulation of bifidobacterial and lactobacilli bacterial populations and discussed in the previous section. Importantly, most of the SCFA production occurred during the first 12h of fermentation. Acetic and propionic acids were the main SCFA produced in all media containing any of the four extracts or FOS. Highest values for both acetic and propionic acids were observed in media containing FOS, yet important concentrations were also produced in media

containing either of the *Ph. nameko* extracts. Propionic acid concentrations were highest for media containing FOS or the *Ph. nameko* extract obtained with Flavourzyme. These concentrations may be related to the high numbers of Bacteroides present or to the presence of specific compounds in the extracts. Broekaert et al. (2011) associated propionic acid production with the side chains found in xilooligosaccharides. An interesting observation is that the acetic-to-propionic ratio increased along fermentation for all substrates except for the *Ph. nameko* Flavourzyme extract, which registered an effective decrease (1.1 to 0.8). This may be important since low acetic-to-propionic ratios have been proposed as a positive marker for a hypolipidemic effect consequence of cholesterol biosynthesis inhibition (Salazar et al., 2008). All tested extracts led to the production of low levels of butyric acid by 24h fermentation in comparison to the positive control FOS (in general two-fold higher); values reported for FOS were however quite moderate in comparison to values reported for other studies (for example 12-24 mM in Gullon et al., 2014) albeit a high variability between donors must be highlighted in this latter case. Related results were reported by Benus et al. (2010) study who showed that butyric acid was reduced following the fibre-supplemented diet (FOS and pea fibre).

Table 9.2. Mean and standard deviation of lactic acid and SCFA (mM) in digested enzymatic extracts of seaweeds and mushroom and the respective controls over 24h of *in vitro* fermentation.

	Time (hrs)	Negative Control	FOS	*S. muticum* Alcalase	O. *pinnatifida* Viscozyme	*Ph. nameko* Flavourzyme	*Ph. nameko* Cellulase
Lactic Acid	0	1.0	-	1.7±0.7	2.4±0.1	6.8±0.7	4.6±3.1
	6	2.9±1.0	12.2±6.2	4.7±0.7	2.9±1.4	8.5±1.5	7.1±0.9
	12	0.5	25.6±4.9	2.8±0.5	1.5±0.5	5.8±2.6	3.9±1.9
	24	1.0	2.2±1.2	3.4	1.0±0.2	2.4	2.2±0.7
Acetic acid	0	0.9	0.9	0.9	1.3±0.4	5.3	0.9
	6	3.8±0.9	10.4±6.4	5.3±0.3	2.7±1.2	7.9±2.1	6.4
	12	6.1±1.4	37.3±20.4	8.2±1.2	6.4±5.1	21.6±2.2	19.2±4.8
	24	7.4±2.9	45.0±5.7	12.5±1.0	11.2±9.1	20.5±11.5	24.9±3.1
Propionic acid	0	-*	-	1.4	-	5.1	-
	6	-	17.1	3.5	4.6	9.8±1.7	7.7±3.1
	12	3.5	36.4	6.4±0.3	5.1	19.4±3.0	15.2±1.3
	24	-	20.6±14.7	7.2±0.7	6.6	24.9±5.7	20.6±4.3
Butyric acid	0	-	-	-	-	-	-
	6	0.3	0.7	-	-	0.7	-
	12	1.8±1.5	0.6±0.2	-	2.5	2.3±1.5	1.6±0.4
	24	5.0	6.6±5.3	0.3	5.9	3.0±2.8	2.3±1.8
Isobutyric acid	0	-	-	-	-	-	-
	6	0.2	-	0.2	0.2	-	-
	12	0.6±0.4	-	0.2	1.7	-	-
	24	1.2	2.6±1.4	-	1.7	1.2±0.6	0.7
Isovaleric acid	0	-	-	-	-	-	-
	6	-	-	-	-	-	-
	12	1.1±0.5	-	-	1.7	1.3	1.0
	24	0.7±0.6	0.3	-	1.3	1.3	2.0

* Lactic acid or SCFA not detected; Values without SD means that SCFA were detected only in one donor.

Concentration of the branched chain fatty acids, iso-butyric and iso-valeric were either below detection limit or in many cases were detected in only one donor.

Lactic acid production was highest when FOS was used as a substrate correlating well with the predominant *Bifidobacterium*/*Lactobacilli* populations. Lactic acid was also produced in the media containing the tested substrates along the first 6h of fermentation, in contrast to medium with

FOS which achieved maximum lactic acid production by 12h fermentation. Thereafter, lactic acid was consumed independently of the substrate in question. Consumption rate was highest in media containing FOS. This observation may eventually suggest a cross-feeding mechanism (Gullon et al., 2014).

The differences observed in SCFA and lactic acid production for the four substrates tested tend to indicate that a relationship may exist between physico-chemical properties of extracts and modulation of individual bacterial species and SCFA production in the gut. As discussed in the previous chapter the four extracts tested displayed different composition and structures some of which may be more accessible for use. The different enzymatic treatments on the different seaweed or mushroom sources lead to the release of different oligomer residues from the structural and storage polysaccharides making these susceptible to degradation. Similarly, Ramnani et al. (2012) showed that low molecular weight extracts derived from agar and alginate seaweeds were fermentable by gut microbiota leading to important increases in acetate and propionate.

9.3.3. Anti-hypertensive activity

A number of ACE inhibitors have been extensively used in the treatment of essential hypertension and heart failure in humans, these include captopril against which novel natural sources are compared in terms of enzyme inhibition capacity. These commercial ACE inhibitor drugs are known to cause side effect such as skin rashes and searching for ACE inhibitors from natural resources has thus become of great relevance. Among these natural resources are marine seaweeds and mushrooms. Hence, the four selected extracts were also screened for their *in vitro* angiotensin -I converting enzyme (ACE) inhibitory activities.

The extract of *O. pinnatifida* obtained with Viscozyme exhibited the strongest ACE inhibitory activity whereas the extract of *Ph. nameko* obtained with Cellulase possessed the second highest ACE inhibitory activity. The IC_{50} values, which are concentrations required to inhibit 50% activity of ACE, for ACE inhibitory activities of these two extracts were 111.2 and 130.4 ug/mL, respectively. The IC_{50} value for ACE inhibitory activity of the *S. muticum* extract obtained with Alcalase was of little significance (444.4 μg/mL) and no inhibition was observed for the extract of *Ph. nameko* obtained with Flavourzyme. Results seem to indicate that the *O. pinnatifida* extract may contain important amounts of active substances capable of inhibiting the ACE activity. There are not many available studies describing the ACE-inhibitory activity of seaweeds extracts. Athukorala & Jeon (2005) reported an IC_{50} value of ca. 0.3 μg/mL for anti-ACE activity of *Ecklonia cava*, while Cha et al. (2006) characterized the ACE inhibitory activities of methanol and aqueous extracts of twenty-six red seaweeds obtained from the coast of Jeju Island in Korea having obtained IC_{50} values that ranged from 12.2-124.7 μg/mL; the value reported for the *O. pinnatifida* extract are within this range. Other studies have shown that *Undaria pinnatifida* peptides decreased blood pressure significantly in spontaneously hypertensive rats (SHRs) after oral administration (Kunio et al., 2004;

Sato et al., 2002). More recently Nishibori et al. (2013) proposed that *Undaria pinnatifida* sporophyll may contain novel non-peptide substances with very important ACE inhibitory activity in vitro (similar to that of captopril), which is an interesting perspective given that most common ACE-inhibitory compounds are of peptide origin.

In what concerns reported ACE-inhibitory activity by mushroom derived extracts, D-mannitol, a major phytochemical of *Pleurotus cornucopiae* was found to inhibit an ACE and lower the blood pressure in SHRs (Hagiwara et al., 2005). No related studies concerning *Ph. nameko* extracts were identified.

9.4. Conclusions

All tested extracts had an influence on composition of human gut microbiota, albeit to different extents. The digested *Ph. nameko* extract obtained with Flavourzyme was found to inhibit growth of *Clostridium histolyticum* and growth of *members* of the *Clostridium coccoides–Eubacterium rectale* group, while growth of *Bifidobacterium* spp. was enhanced and *Lactobacillus* spp. remained relatively unaffected. This selective increase in bifidobacteria coupled to a consistent increase in total SCFA and lactic acid production suggest its potential prebiotic character.

Seaweed extracts, in particular that of *O. pinnatifida* obtained with Viscozyme, were fermentable by gut microbiota as indicated by an increase in SCFA. Increase in SCFA was not always correlated with an increase in bacterial populations for the seaweed extracts.

Despite lack of selectivity concerning prebiotic potential of *O. pinnatifida* extract, it was found to have a moderate ACE inhibitory activity.

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Chapter 10

Development of a functional food by incorporation of the selected extracts with biological properties

10.1. Introduction

Fortification of foods with functional ingredients that may impact on specific functions or systems in the human body, providing health benefits beyond nutritional value has become an increasingly popular method and target of many studies over the last years (Kadam & Prabhasankar, 2010; Prabhasankar et al., 2009). This growth is fuelled by technological innovations, development of new products, and the increasing number of health-conscious consumers interested in products that improve life quality. The increasing ageing of populations, the decrease in quality of life due to stress, the high incidence of the so-called modern diseases (cardiovascular disease, obesity, cancer, diabetes, and allergies) represent driving forces in the quest for different foods and diets to promote healthy active ageing, improve well-being and to counteract the incidence of many diseases.

Seaweeds and mushrooms as previously discussed represent a tremendous potential as sources for many new healthy functional food ingredients and biologically active compounds constituting a research area with much to explore for food proposes. On the other hand, many foods already widely consumed all over the world are a potential vector for incorporating new functional ingredients from marine algae or mushrooms, which will enhance their value at both nutritional and economic levels (Cha et al., 2013; Ulziijargal et al., 2013; Cofrades et al., 2011; Sim et al., 2011; Kim & Joo, 2010). The development of foods that promote general health and wellbeing has been a research priority for food industry; foods enriched with such healthy compounds are indeed sought to further and further extents. Novel functional products that combine existing nutritional richness with important health promoting features are thus in order, especially if they are organoleptically appealing (Madureira et al., 2015).

Considering the potential of a dairy vector for incorporation of the 4 selected seaweed and mushroom extracts and inspired on new dairy products in the market as well as in the previous experience in the dairy field with probiotic and synbiotic dairy matrices (Rodrigues et al., 2012, 2011a,b), efforts were made to produce a healthy, versatile dairy matrix based on whey cheese (*Requeijão*) and yoghurt producing a spreadable dairy cream. *Requeijão* which is based on heat-precipitated proteins from whey, contains moderate fat levels (in the range 8–14%, by mass) coupled with several proteins such as α- lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase, serum albumin and glycomacropeptide which are acclaimed for their nutritional and health-related features such as immune system stimulation and anti-carcinogenic activity (Madureira et al., 2015, 2008). Complimentarily, yoghurt has an outstanding position in the development of functional foods given its natural physiological benefits besides its positive organoleptical features, freshness resulting from the fermentation by added lactic acid bacteria. There has been a considerable increase in the popularity of yoghurt in recent years and the incorporation of functional ingredients therein can add extra nutritional and physiological values.

In this chapter the main objective was to develop a new functional food based on a dairy spreadable cream with incorporated extracts based on the assessment of their biological and

technological potential. Biochemical characterization, sensorial evaluation, characterization and assessment of *in vitro* stability of functional extracts as well as storage stability are presented.

10.2. Material and methods

10.2.1. Assessment of functionality of a dairy matrix

10.2.1.1. Production of spreadable dairy cream

As described in the introduction section, it was decided to produce a versatile and healthy dairy matrix based on whey cheese (*requeijão*) and yoghurt producing a spreadable dairy cream. Pasteurized whey cheese from cows' milk and a Greek type yogurt (without sugar) were obtained from the local supermarket and different concentrations of whey cheese, yoghurt and extracts were tested (data not shown). According to the best flavour and aroma characteristics, evaluated by laboratory internal staff, the best recipe for the spreadable dairy cream, and that chosen for further studies, was based on 75% (w/w) of pasteurized whey cheese, 22% (w/w) of Greek type yogurt and 3% (w/w) of seaweed or mushroom extract (Figure 10.1).

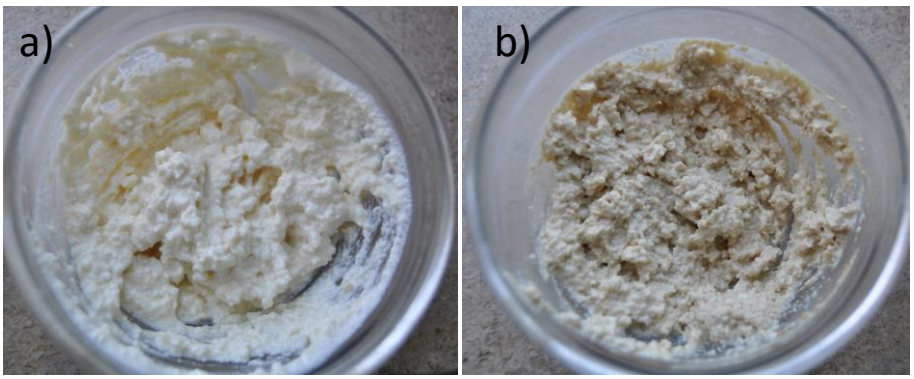

Figure 10.1. Samples of spreadable dairy cream incorporating enzymatic extract of *O. pinnatifida* obtained with Viscozyme (a) and of *Ph. nameko* obtained with Flavourzyme (b).

10.2.1.2. Prebiotic activity of spreadable dairy cream with incorporated extracts

In order to evaluate the biological potential of the spreadable dairy cream incorporating the four selected seaweed and mushroom extracts, assays with pure cultures of *L. acidophilus* La-5 and *B. animalis* BB12 were performed in order to monitor the potential prebiotic activity. Assessment was performed as described in chapter 5 (section 5.2.5) with some modifications. The numbers of viable cells of *L. acidophilus* La-5 and *B. animalis* BB12 were evaluated in six different spreadable dairy creams over 48 hours incubation at 37 ºC: four spreadable dairy creams with incorporated

seaweed or mushroom extract (3%, w/w), one spreadable dairy cream with 3% (w/w) FOS (positive control) and a spreadable dairy cream without addition of a carbon source (negative control). These spreadable dairy creams were inoculated with 2% (v/w) of *L. acidophilus* La-5 or *B. animalis* BB12. To obtain each probiotic inoculum, freeze dried bacteria of *B. animalis* BB12 or of *L. acidophilus* La-5 (CHR-Hansen, Denmark) were inoculated twice in MRS broth (Biokar Diagnostics, France); 2% (v/v) of a 24-h probiotic culture was then inoculated in MRS broth supplemented with 0.5 g L^{-1} L-cysteine-HCl (Panreac, Spain) in 50 mL flat-bottomed glass flasks, and incubated for another 24h.

Each inoculated spreadable dairy cream was divided into 5 portions of 4 g each and aseptically placed in containers, to be incubated at 37 ºC and sampled at 0, 6, 12, 24 and 48h. Two replicas were performed for each spreadable dairy cream.

For each spreadable dairy cream sample and sampling point, a 2.4 g aliquot was homogenized in 24 mL of sterile 2% (w/v) sodium citrate (Fisher Scientific, UK) for 3 min, in a Stomacher blender (Model 400 circulator, from Seward Laboratory Systems, UK). Sequential dilutions were made, with sterile 0.1% (w/v) peptone water (Himedia) of spreadable dairy cream homogenates and 20 µL aliquots were plated, in duplicate, on Petri dishes of MRS agar (Biokar Diagnostics). Viable cell numbers of probiotic bacteria were obtained in MRS agar containing 0.5 g/L L-cysteine- HCl, following incubation at 37 ºC for 48h, in an anaerobic cabinet for *L. acidophilus* La-5 and *B. animalis* BB12 at 0, 6, 12, 24 and 48h

10.2.2. Development of a functional food by incorporation of two extracts with higher biological potential

10.2.2.1. Production of spreadable dairy cream

Considering the results from the prebiotic activity assessment of spreadable dairy cream incorporated with the four different seaweed and mushroom extracts, it was selected the most two promising ones to move further in the studies and development of a new functional dairy food.

In this stage some assays were performed to increase the shelf-life of whey cheese which is known to be a relatively perishable food product with limited shelf life. Since no time and opportunity was found to produce the whey cheese at the laboratory facilities, it was chosen to apply some thermal treatments to the whey cheese bought in the local market.

Whey cheese was subjected to thermal processing either in an autoclave at 110 ºC for 10 minutes or in an agitated water bath at 90 ºC for 10 min. The heated samples were then evaluated in terms of microbiological safety before and after thermal processing. Sequential dilutions of heated whey cheese were made with sterile 0.1% (w/v) peptone water and viable cell numbers were enumerated in Plate Count Agar (Himedia) incubated aerobically at 30 ºC for 48h. No viable cell numbers were detected in -1 to -5 dilutions after incubation for both heat treatments.

Upon microbiological stability assessment the final formulation decided for the spreadable dairy cream was its production with 75% (w/w) whey cheese thermally processed at 90 ºC for 10 min, with 22% (w/w) of greek type yogurt and 3% (w/w) of extract.

10.2.2.2. Microbiological stability under refrigerated storage

Duplicate spreadable dairy creams were produced with each extract, divided in portions of 6 g and placed aseptically in containers and stored at 5±1 ºC over a period of 14 days. Microbiological analyses were performed at 0, 7 and 14 days of storage.

For each spreadable dairy cream and sampling time a 2.4 g aliquot of each spreadable cream was homogenized in 24 mL of sterile 2% (w/v) sodium citrate for 3 min, in a Stomacher blender. Sequential dilutions were made with sterile 0.1% (w/v) peptone water, of spreadable dairy cream homogenates and 20 µL aliquots were plated, in duplicate, on Petri dishes of MRS agar, PCA and Violet Red Bile Glucose Agar (VRBGA, Biokar Diagnostics) and incubated aerobically at 37 ºC for 48h.

10.2.2.3. Prebiotic activity

In order to confirm the biological potential of the spreadable dairy cream incorporating the extracts, assays with pure cultures of *L. acidophilus* La-5 and *B. animalis* BB12 were performed according to the procedures described above in section 10.2.1.2.

10.2.2.4. α-Glucosidase inhibitory activity

The evaluation of the α-Glucosidase inhibitory activity in samples of spreadable dairy cream with incorporated extracts was performed according to procedure described in chapter 5 section 5.2.5. However due to the presence of proteins and some fat in the dairy matrix it was not possible to measure adequately the enzyme activity.

10.2.2.5. pH, glucose and organic acid analysis

The pH of the spreadable dairy cream samples was measured directly with a pH meter (Micro pH 2002, Crison, Spain). Duplicate samples of each spreadable dairy cream were assessed for glucose and organic acids (lactate and acetate) by HPLC in a single run, based on calibration curves previously prepared with appropriate chromatographic standards, using an apparatus from Merck LaChrom (Fullerton CA, USA), with and Aminex HPX-87X cation exchange column from BioRad (Richmond CA, USA); the flow rate was 0.8 mL min^{-1}; 13 mM H_2SO_4 (Merck, USA) was used as eluant; and detection was by refractive index at 65 ºC for glucose, and UV absorbance at 220 nm for organic acids. Prior to analysis, all samples were pre-treated as follows: 1 g of

spreadable dairy cream was homogenized with 5 mL of 13 mM H_2SO_4 in an Ultra-Turrax (IKA®, Canada) at 4000 rpm, allowed to stand for 3 min in an ice-bath, centrifuged at 14 000 rpm for 10 min at 4 ºC, and then filtered through a 0.22 µm membrane filter (Millipore, USA).

10.2.2.6. Sensorial Analysis

A sensory panel assessed three coded experimental spreadable dairy creams at random. Spreadable dairy creams evaluated included those with each seaweed and mushroom extract and without addition of carbon source. The panel consisted of 16 members, specifically trained for dairy product organoleptic analyses, with average age of 34±9 mainly Portuguese (94%) female (88%), 50% of which indicated regular consumption of whey cheese several times in a month. Spreadable dairy creams were placed into air tight plastic containers, and conditioned at room temperature for 15 min before evaluation. Between analyses, the panel took water and ”granny smith” apple, so as to eliminate the taste of the previous analysis.

Consumer acceptance was measured by overall liking ratings, provided on a 9-point hedonic scale (Peryam & Girardot, 1952; Peryam & Pilgrim, 1957), whereas the consumption intent was evaluated in a probability scale ranging from 0 to 10 based on Juster (1966). During the essay, the intensity of aspect, odour, flavour, creaminess and consistency sensory attributes were measured by ratings provided on a just-about-right scale (Moskowitz, 1972).

10.2.3. Evaluation of stability of bioactive compounds after simulated digestion of spreadable dairy cream with incorporated seaweed extract

A spreadable dairy cream with extract of *O. pinnatifida* obtained with Viscozyme was prepared and was subject to simulated gastrointestinal digestion according to procedures described in chapter 9, section 9.2.2.1.1. Upon digestion, retentate liquid from dialyses membrane were frozen at –80 ºC and lyophilized.

Lyophilized retentate was evaluated for prebiotic activity with pure cultures (*L. acidophilus* La-5 and *B. animalis* BB12) according to procedures described in chapter 5, section 5.2.6 and for α-Glucosidase inhibitory activity, performed according to procedure described in chapter 5, section 5.2.5. However due to the interferences from nutrients in the sample it was not possible to measure the enzyme activity.

10.2.4. Statistical Analysis

Data are expressed as the mean plus standard deviation of replicates. For prebiotic activity and for each probiotic bacterium, a two-way ANOVA was carried out with SigmaStat™ (Systat Software, Chicago, IL, USA), to assess whether the extract and incubation time at 37 ºC were significant sources of variation for the bacterial viable cell numbers and pH values. Since significant

differences were observed for each factor (extract type and incubation time) as well as significant interactions (p<0.05), one-way ANOVAs were carried out in order to observe if the carbon source (extract or FOS or negative control) were statistically significant (p<0.05) for the number of viable cells of *L. acidophilus* La-5 or of *B. animalis* BB12 and for pH, after 24 or 48h of incubation or after 10 and 24h, respectively. One-way ANOVAS were also performed to evaluate statistical significance (p<0.05) of the viable cell numbers after 24 and 48h or after 10 and 24h, in comparison to values at 0h.

For sensorial analysis, the existence of significant differences between samples in term of consumer acceptance and consumption intent was assessed using Wilcoxon signed-rank test. The significance of statistical tests was evaluated at p<0.05.

10.3. Results and discussion

10.3.1. Assessment of functionality of a dairy matrix

The main evaluation in terms of functionality was based on prebiotic potential of the spreadable dairy cream incorporating the different seaweed and mushroom extracts. In Figures 10.2 and 10.3 it is displayed the variation of viable cell numbers of *B. animalis* BB12 and *L. acidophilus* La-5 over 48h as well as respective variation of pH. Spreadable dairy cream with extract of *S. muticum* was responsible for lower counts of both *B. animalis* BB12 and *L. acidophilus* La-5, in particular after 12 h of incubation (Figures 10.2a and c). Better performance is observed for spreadable dairy cream with extract of *O. pinnatifida* in particular for *L. acidophilus* La-5 being responsible for statistically significant (p<0.05) higher values after 12 and 24h of incubation and in comparison to control spreadable dairy creams. For *B. animalis* BB12 counts, the differences between spreadable dairy cream with extract of *O. pinnatifida* and both controls were not statistically significant (p>0.05). Higher increment ratio of viable cell numbers *of B. animalis* BB12 in comparison to time 0h is observed in spreadable dairy cream with *O. pinnatifida* at 12h.

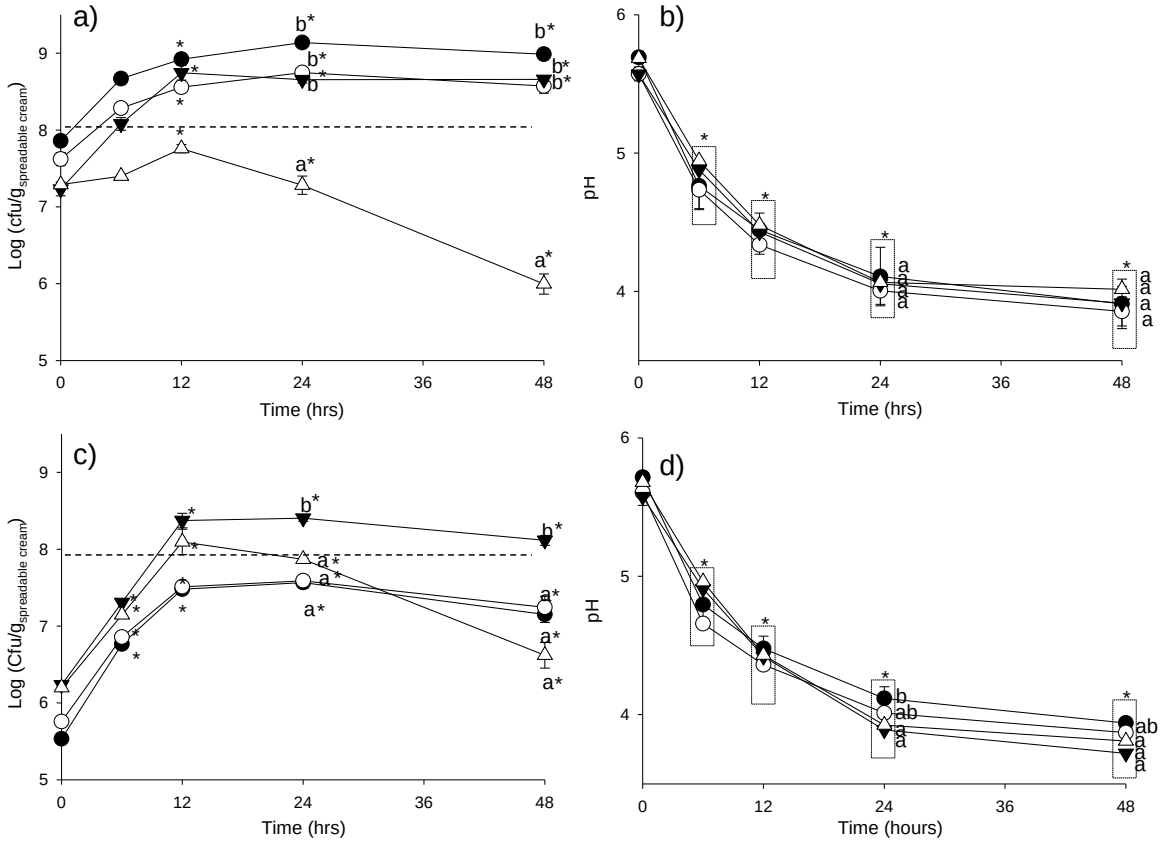

Figure 10.2. Variation of viable cell numbers of *B. animalis* BB12 (a) and of *L. acidophilus* La-5 (c) and respective pH (b and d) in spreadable dairy cream incorporated with *S. muticum* obtained with Alcalase (△), *O. pinnatifida* obtained with Viscozyme (▼), FOS (●) and without any carbon source (○) over 48 h of incubation at 37 ºC. Different letters indicate significant differences (p<0.05) for the viable cell numbers after 24 or 48h of incubation at 37 ºC in the presence or absence of carbon sources (extracts and FOS) and * for significant differences (p<0.05) in comparison to values at 0h.

Both spreadable dairy cream and time factors were statistically significant factors (p<0.05) for pH variation over 48h, except for pH variation in spreadable dairy creams inoculated with *B. animalis* BB12 where only incubation time was the significant factor (Figures 10.2b and d); the initial pH ranged from 5.6-5.7 at 0h to 3.7-4.0 at 48h for all samples. Spreadable dairy creams inoculated with *L. acidophilus* La-5 and incorporating both *S. muticum* and *O. pinnatifida* extracts presented slightly lower values of pH after 24 and 48h (Figure 10.2d). Although lower values of pH may bring about greater loss in viable cell numbers of probiotic strains this was not the case in the spreadable dairy creams containing the different extracts except for that containing the *S. muticum* extract.

Spreadable dairy creams with both *Ph. nameko* extracts evidences higher prebiotic activity in particular with enzymatic extract obtained with Flavourzyme for both probiotic bacteria (Figures 10.3a and c). At 24 and 48h statistically significant differences (p<0.05), higher than 1 cycle log, were observed for counts of *L. acidophilus* La-5 in spreadable dairy creams with both enzymatic

extracts of *Ph. nameko* in comparison to spreadable dairy cream with FOS and negative control (Figure 10.3c). The Flavourzyme extract was also responsible for statistically significant (p<0.05) higher viable cell numbers of *B. animalis* B12 in comparison to Cellulase extract and both controls at 24 and 48h (Figure 10.3a). Recall that higher viable cell numbers above 8 log cfu/mL of *L. acidophilus* La-5 and *B. animalis* BB12 were also recorded for MRS with 6% of both enzymatic extracts with Cellulase and Flavourzyme (Chapter 7, Table 7.2).

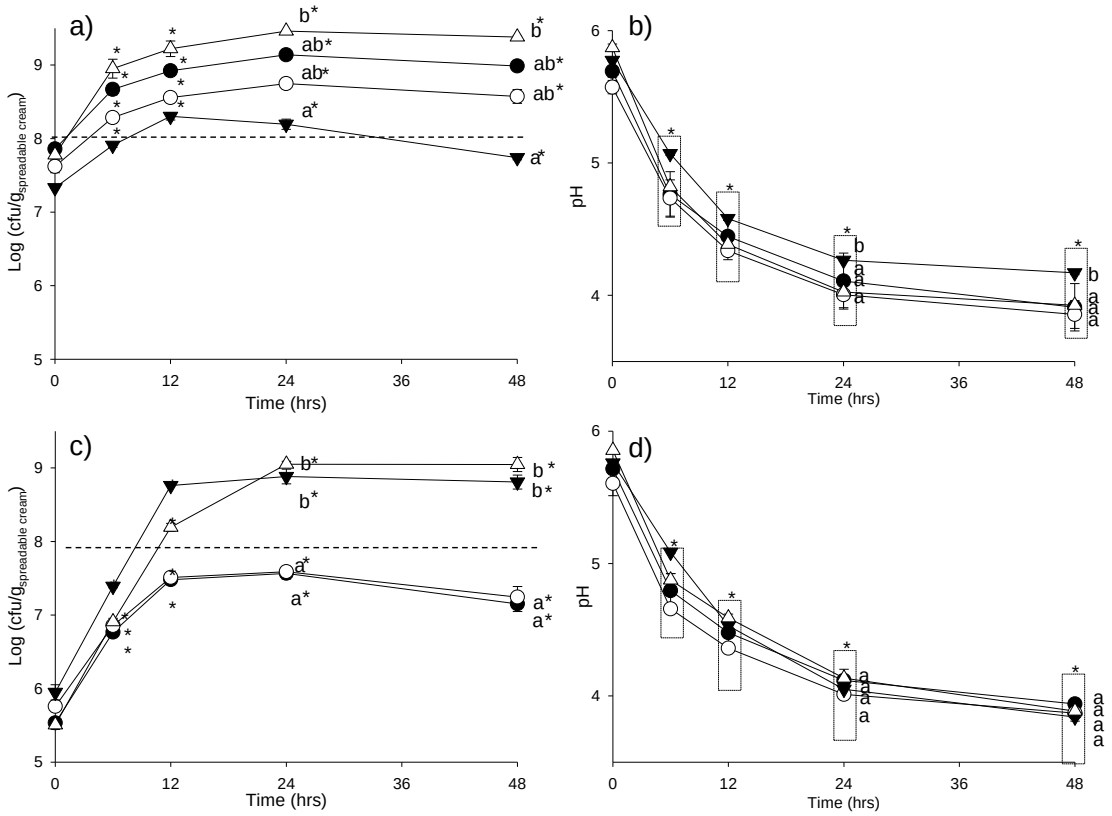

Figure 10.3. Variation of viable cell numbers of *B. animalis* BB12 (a) and of *L. acidophilus* La-5 (c) and respective pH (b and d) in spreadable dairy cream incorporated with *Ph. nameko* obtained with Cellulase (▼), *Ph. nameko* obtained with Flavourzyme (△), FOS (●) and without any carbon source (○) over 48h of incubation at 37 ºC. Different letters indicate significant differences (p<0.05) for the viable cell numbers after 24 or 48h of incubation at 37 ºC in the presence or absence of carbon sources (extracts and FOS) and * for significant differences (p<0.05) in comparison to values at 0h.

Both spreadable dairy cream and time factors were statistically significant factors (p<0.05) for pH variation over 48h (Figures 10.3b and d); the initial pH ranged from 5.6-5.7 at 0h to 3.7-4.0 at 48h for all samples. Spreadable dairy creams inoculated with *B. animalis* BB12 incorporating Cellulase *Ph. nameko* extracts presented a pH profile with statistically significant differences at 24 and 48h, in comparison with the other spreadable dairy creams (Figure 10.3b).

In general, the spreadable dairy cream without addition of extra carbon source promoted survival and growth of both *B. animalis* BB12 and *L. acidophilus* La-5. In particular for *L. acidophilus* La-5, these differences were comparable and not statistically different in relation to spreadable dairy cream with FOS (Figures 10.2c and 10.3c), which indicates that *per se* that the spreadable dairy cream composed of whey cheese and yoghurt is a suitable matrix vector for probiotic bacteria. This finding is not surprising and is in agreement with previous works on probiotic whey cheese (Madureira et al., 2015, 2008) or even symbiotic cheese matrices (Rodrigues et al., 2011b). Therefore the increments of growth and survival due to the presence of 3% (w/w) of the extracts are a promising result in terms of prebiotic activity potential of extracts. Higher values than 7 log cfu/mL of *L. acidophilus* La-5 and *B. animalis* BB12 was recorded for MRS with 6% of both enzymatic extracts of *S. muticum* and *O. pinnatifida* (Chapter 5, Figures 5.2 and 5.3) and for both enzymatic extracts of *Ph. nameko* (Chapter 7, Table 7.2).

Based on the results obtained for growth and stability of pure cultures of *L. acidophilus* La-5 and *B. animalis* BB12 with the spreadable dairy creams as well as those obtained with *in vitro* fermentations presented and discussed in chapter 9 with the seaweeds and mushrooms extracts, the extract of *O. pinnatifida* obtained with Viscozyme and the *Ph. nameko* obtained with Flavourzyme were selected for further studies on confirmation of the potential of the new dairy food.

10.3.2. New functional dairy food by incorporation of two extracts

New spreadable dairy cream with both selected extracts was produced with whey cheese previously subjected to thermal processing, as described in section 10.2.2.1, added with yoghurt, and stored under refrigerated conditions, at 5±1 ºC, over 14 days.

Considering the microbiological stability and the visible changes (texture and colour) of the whey cheese upon both thermal processing treatments it was selected to submit the whey cheese to heat treatment in an agitated water bath at 90 ºC for 10 min. Whey cheese heat-treated in an autoclave at 110 ºC for 10 minutes turned grainy, due to some protein precipitation, and suffered some caramelization which evidenced nutritional losses. Whey cheese heat-treated in an agitated water bath after 90 ºC for 10 min did not show any textural and colour changes. During this process the inner temperature of the whey cheese oscillated between 70 and 75 ºC.

Microbiological control of spreadable dairy creams with incorporated extracts as well with FOS and without addition of carbon source was made at 0, 7 and 14 days of storage (Table 10.1). The appearance of relatively high amounts of viable cell numbers of *Enterobacteriaceae* at 7 days of storage, confirmed by the viable cell numbers in PCA, revealed that probably the thermal processing selected was not sufficiently effective to assure no microbiological growth in the dairy matrix or, in alternative, the manipulations during the production of the spreadable dairy creams were not able to avoid contaminations. Microbiological counts at 14 days of storage confirmed the levels of contamination observed at 7 days although the pH variation was not as pronounced at 14

days of storage in comparison to 7 days (Table 10.1). These results are an indication that, currently, the newly developed spreadable dairy cream has no more than 5 or 6 days of shelf-life and more efforts have to be made to increase its shelf life at least to 14 days in order to guarantee product stability.

In the sequence of the results obtained from the microbiological control, only spreadable dairy creams immediately upon their production were evaluated for prebiotic activity with evaluation of production of organic acids and antidiabetic activity whereas spreadable creams with two days of storage were evaluated in terms of sensorial properties.

Table 10.1. Mean and standard deviation of viable cell numbers [Log (cfu/$g_{spreadable\ cream}$)] grown on PCA, VRBGA and MRS from spreadable dairy creams without addition of carbon source and with extracts of *O. pinnatifida* obtained with Viscozyme, *Ph. nameko* obtained with Flavourzyme and FOS over 14 days of storage under refrigerated conditions.

Spreadable cream	Time (days)	Log (cfu/$g_{spreadable\ cream}$)			pH
		PCA	VRBA	MRS	
Without Carbon source	0	> 2.7	nd[1]	nd[2]	5.92±0.03
	7	> 5.7	6.6±0.1	7.21±0.01	4.84±0.02
	14	8.31	8±0.1	7.29±0.09	5.3±0.8
FOS	0	> 2.7	nd[1]	nd[2]	5.83±0.01
	7	> 5.7	6.52±0.04	<6.7	4.86±0.01
	14	>7.7	7.36±0.01	7.18±0.01	5.4±0.2
O. pinnatifida Viscozyme	0	> 2.7	nd[1]	nd[2]	5.84±0.01
	7	> 5.7	> 6.7	7.07±0.01	5.48±0.01
	14	7.9±0.1	7.36±0.01	7.65±0.09	5.4±0.2
Ph. nameko Flavourzyme	0	> 2.7	nd[1]	nd[2]	5,85±0.01
	7	> 5.7	6.43±0.01	7.22±0.01	5.04±0.02
	14	8.1±0.1	7.9±0.2	7.87±0.01	5.00±0.08

[1]Not detected *Enterobacteriaceae* cells in the first decimal dilution; [2]Not detected aerobic lactobacilli cells in the first decimal dilution.

The evaluation of prebiotic potential of the spreadable dairy cream incorporating the *O. pinnatifida* and *Ph. nameko* extracts was repeated with pure cultures of *B. animalis* BB12 and *L. acidophilus* La-5 over 24h (Figure 10.4a and c). The variations in pH as well as of glucose consumption and organic acids production were recorded over the 24h and are displayed in Figures 10.4.b-d and in Table 10.2, respectively.

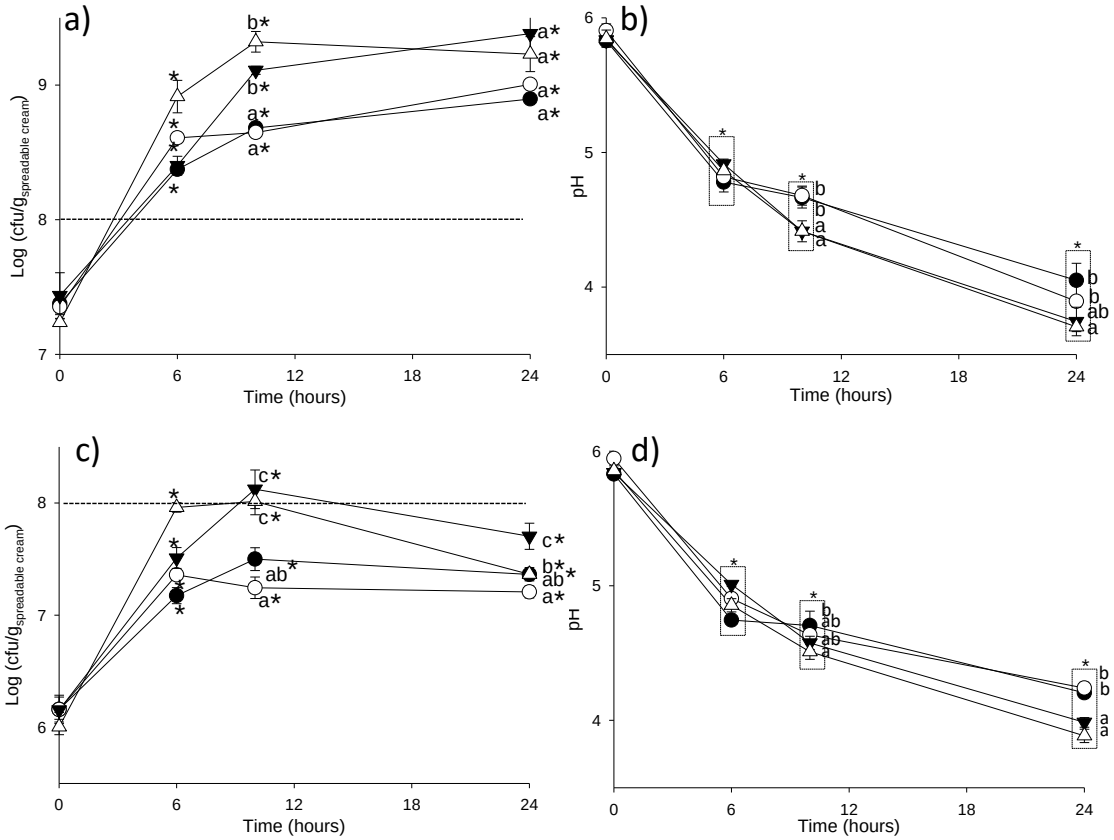

Figure 10.4 Variation of viable cell numbers of *B. animalis* BB12 (a) and of *L. acidophilus* La-5 (c) and respective pH (b and d) in spreadable dairy cream incorporated with *O. pinnatifida* obtained with Viscozyme (▼), *Ph. nameko* obtained with Flavourzyme (△), FOS (●) and without any carbon source (○) over 24h on incubation at 37 ºC. Different letters indicate significant differences (p<0.05) for the viable cell numbers after 12 or 24h of incubation at 37 ºC in the presence or absence of carbon sources (extracts and FOS) and * for significant differences (p<0.05) in comparison to values at 0h.

Spreadable dairy creams with both extracts confirmed their prebiotic potential promoting statistically significant increments (p<0.05) of viable cell numbers for both probiotic bacteria under study which is particularly evident at 10 h of incubation (Figures 10.4a and d) in comparison to FOS and negative control. Interesting values of viable cells were observed for *B. animalis* BB12 which increased around 2 log cycles between 0 and 10h of incubation attaining values of 9.1 to 9.3 log (cfu/$g_{spreadable\ cream}$), which were maintained until 24h of incubation [9.1 to 9.3 log (cfu/$g_{spreadable\ cream}$)]. Both spreadable dairy cream and time factors were statistically significant factors (p<0.05) for pH variation over 24h (Figures 10.4b and d). Slightly lower values of pH were observed for both spreadable dairy creams with extracts in comparison to FOS and negative control at 10 and 24h, both for *B. animalis* BB12 as for *L. acidophilus* La-5. These values are certainly related with the higher content of lactic acid in the spreadable dairy creams with extracts at 10 and 24h in comparison to FOS and negative control (Table 10.3); the differences of lactic acid content

between both spreadable dairy creams with extracts were not statistically significant (p>0.05) but higher and statistically significant than spreadable with FOS and negative control (p<0.05). Significant increases in lactic acid were recorded over the 24h of incubation for all spreadable dairy creams. Acetic acid was only detected after 24h of incubation in all spreadable dairy creams with slightly higher values, but not statistically significant, for the spreadable dairy creams with extracts, in particular for those inoculated with *B. animalis* BB12. Glucose, in turn, present as trace free residues from both FOS and extracts followed a comparable trend; concentrations diminished steadily over the 24 hour incubation period independently of the spreadable dairy cream considered.

Table 10.2. Mean and standard deviation of glucose, lactic acid and acetic acid ($\mu g/g_{spreadable\ cream}$) in spreadable dairy creams, without addition of carbon source and with extracts of *O. pinnatifida* obtained with Viscozyme, *Ph. nameko* obtained with Flavourzyme and FOS, inoculated with *B. animalis* BB12 and *L. acidophilus* La-5 throughout 24h of incubation at 37 ºC for evaluation of prebiotic activity.

	Time (hrs)	No Sugar			FOS			*O. pinnatifida*_Visc			*Ph. nameko*_Flav		
		Glucose	Lactic acid	Acetic acid	Glucose	Lactic acid	Acetic acid	Glucose	Lactic acid	Acetic acid	Glucose	Lactic acid	Acetic acid
L. acidophilus La-5	0	2.1±0.2	4.2±0.2	-*	2.3±0.4	4.2±0.1	-	2.4±0.4	4.7±0.1			5.6±0.1	-
	6	1.6±0.3	9.22±0.02	-	2.4±0.1	9.6±0.2	-	2.2±0.5	7.9±0.2	-	2.04±0.03	11.2±0.1	-
	10	1.3±0.1	9.9±0.1	-	0.1±0.2	11.0±0.2	-	1.6±0.1	11.1±0.2	-	1.63±0.02	14.7±0.1	-
	24	1.5±0.2[a]	14±2[a]	2.5±0.0[a]	2.14±0.02[a]	13.86±0.03[a]	2.5±0.0[a]	1.9±0.3[a]	19.2±2.9[b]	2.72±0.02[a]	1.5±0.1[a]	20.5±0.6[b]	2.78±0.03[a]
B. animalis BB12	0	1.9±0.4	4.4±0.1	-	2.4±0.5	4.6±0.1	-	2.3±0.7	4.8±0.3	-	1.9±0.3	5.71±0.04	-
	6	1.873±0.003	10.33±0.07	-	2.0±0.3	10.2±0.1	-	1.9±0.2	8.7±0.1	-	1.99±0.04	12.0±0.3	-
	10	1.74±0.06	12.4±0.1	-	2.2±0.2	12.3±0.7	-	2.0±0.2	15.1±1.6	-	1.60±0.01	17.6±0.3	-
	24	1.4±0.2[a]	16.8±0.2[a]	2.57±0.03[a]	2.5±0.4[a]	19.1±3.1[a]	2.6±0.0	1.60±0.02	22.9±0.1[b]	3.0±0.1[a]	1.5±0.1[a]	26.6±1.1[b]	3.11±0.01[a]

*not detected; Different letters for glucose, lactic acid or acetic acid at 24 h indicate significant differences ($p<0.05$) between spreadable creams.

Spreadable dairy creams incorporating seaweed and mushroom extracts as well as spreadable dairy cream without addition of carbon source were submitted to sensorial evaluation and data collected for consumer acceptance, consumption intention and sensorial attributes intensity are displayed in Table 10.3. Product appraisal to identify specific sensory attributes driving product acceptance is vital to the introduction of new functional dairy products in the market place. Application of an adequate sensory methodology enables one to obtain important results on the formulated dairy foods, providing prior knowledge with respect to its acceptance and/or specific characteristics or a descriptive sensory profile, serving as the foundation for making alterations, when required.

Table 10.3. Sensorial evaluation of spreadable dairy creams

Sensorial Test	Spreadable cream		
	O. pinnatifida Viscozyme	*Ph. nameko* Flavourzyme	Control (without addition)
Consumer acceptance (average±SD)[1]	6.0±2.3	7.3±1.5	7.6±1.1
Positive evaluation	75%	81%	94%
Negative evaluation	25%	6%	0%
Indifferent evaluation	0%	13%	6%
Consumption intention (average±SD)[2]	4.9±3.9	7.1±2.7	7.6±2.0
Attributes intensity (average values)[3]			
Aspect	0.3	0.4	0.6
Odour	0.4	0.4	0.6
Flavour	0.1	0.6	0.6
Creaminess	0.4	0.5	0.7
Consistency	0.5	0.6	0.6

[1]6- Slightly like; 7- Like; 8- Like very much; [2]5- Average chance; 6- Good chance; 7- Probably; 8- Very likely; [3]Intensity measured between -1 to +1.

The spreadable dairy cream with *O. pinnatifida* extract was the food sample that received lowest scores in terms of consumer acceptance and consumption intention being classified by the panel as a “slightly likeable” matrix with an average chance to be consumed. The flavour and general aspect were the sensorial attributes that least pleased the panel. The spreadable dairy cream with *Ph. nameko* extract had higher sensorial scores comparable to the spreadable dairy cream control. Both were classified by the panel between “like” and “like very much” in terms of consumer acceptance whereas for consumption intention, their classification was similarly between probably and very likely (Table 10.3). According to Wilcoxon signed-rank test, no statistical significant differences were observed between spreadable dairy cream with *Ph. nameko* and spreadable dairy cream control both in terms of consumer acceptance (p=0.233), meaning that both spreadable creams were equally appreciated by the panel, and of equal consumption intention (p=0.525), meaning that panel revealed similar intention to consume both spreadable dairy creams. Statistical significant differences were recorded between spreadable dairy cream with *O. pinnatifida* extract and spreadable dairy cream control both in terms of consumer acceptance (p=0.013), meaning that spreadable dairy cream control was better appreciated than spreadable dairy cream with *O. pinnatifida* extract by the panel, and of consumption intention (p=0.023), meaning that

panel revealed higher intention to consume spreadable dairy cream control than spreadable dairy cream with *O. pinnatifida* extract. The liking of the spreadable dairy cream control is naturally expected given the positive characteristics of each of the dairy products used. In a similar line of research Antunes et al. (2005) assessed the impact on acceptance and sensorial attributes of the addition of whey protein concentrate to yogurt with *L. acidophilus* and *B. longum*. A group of 30 subjects assessed the product using a 9-point non-structured hedonic scale to evaluate the degree of liking of appearance, flavour, texture and overall appreciation. Results showed that whey protein added yogurt did not differ significantly (p>0.05) from control and had good sensory acceptance among consumers.

Regardless of the fact that spreadable dairy cream control was, in general, better classified in terms of sensorial evaluation the scores obtained for spreadable dairy cream with *Ph. nameko* extract are still very promising with an important potential of being accepted by regular consumer of dairy products such as spreadable cheeses already existent in the market; furthermore, if associated functional properties are highlighted consumers may expectedly level their degree of acceptance and intention to purchase. In terms of spreadable dairy cream with *O. pinnatifida* extract the obtained scores indicate that some improvement is effectively needed for example, by masking the more astringent flavour by microencapsulation of the added extracts or by combination with other flavouring agents it would turn this product acceptable to be consumed.

To my knowledge no similar dairy food has being studied with incorporation of seaweeds or mushroom extracts and therefore comparisons are not possible. As described in chapter 2, in literature works incorporating seaweeds, mushrooms or ingredients include pasta, bread, meat, milk and fruit juices but none have studied their functionality in terms of prebiotic activity.

10.3.3. Characterization and assessment of *in vitro* stability

A spreadable dairy cream with extract of *O. pinnatifida* obtained with Viscozyme was prepared and it was subject to simulated gastrointestinal digestion to assess its *in vitro* stability. It is well established that any food to be entitled a prebiotic food needs to ensure that functional prebiotic ingredient is not digested before reaching the large intestine. Dairy matrices, in general, are protective toward this requirement but validation is always required for each novel ingredient tested. Viable cells of pure cultures of *L. acidophilus* La-5 and *B. animalis* BB12 were recorded throughout 24h of incubation at 37 ºC in the presence or absence of 6% (w/v) glucose, FOS or the lyophilized digested spreadable dairy cream with extract of *O. pinnatifida* obtained with Viscozyme (Table 10.4). Different potential to promote growth and stability of both probiotic bacteria was observed with the digested spreadable dairy cream in comparison to FOS and glucose positive controls. For *L. acidophilus* La-5, the digested spreadable dairy cream was able to support its survival and growth, in particular after 24h. Statistically significant (p<0.05) lower values of *L. acidophilus* La-5 were obtained with FOS after 24h. In terms of *B. animalis* BB12 growth and stability, a lower potential was observed for the digested spreadable dairy cream in

comparison to FOS and glucose positive controls; viable cell numbers decreased ca. 1 log cycle a trend similar to that observed for the negative control. These results indicate a possible partial degradation of the bioactive compounds in the extract leading to a loss in prebiotic activity. It is important to recall that stability is being verified against two pure probiotic strains, but it should in fact be verified within a natural gut environment as done for the extracts in chapter 9. Albeit the lower numbers there is space for matrix improvement toward prebiotic potential maintenance.

Table 10.4. Mean and standard deviation of cell counts [Log (cfu/mL)] of *L. acidophilus* La-5 and *B. animalis* BB12 throughout 24h of incubation at 37 ºC in the presence or absence of glucose, with FOS or with lyophilized digested spreadable dairy cream with extract *O. pinnatifida obtained with* Viscozyme.

	Time (hrs)	No Sugar		Glucose		FOS		Digested dairy cream	
		Log (cfu/mL)	R*	Log (cfu/mL)	R*	Log (cfu/mL)	R*	Log (cfu/mL)	R*
L. acidophilus La-5	0	6.02±0.02	1.00	6.05±0.01	1.00	5.96±0.04	1.00	5.94±0.01	1.00
	6	6.35±0.04*	1.06	6.3±0.1*	1.05	6.42±0.08*	1.08	6.11±0.09	1.03
	10	7.0 ± 0.2^{b}*	1.17	7.56 ± 0.09^{c}*	1.25	7.618 ± 0.004^{c}*	1.28	6.25 ± 0.08^{a}	1.05
	24	6.42 ± 0.01^{a}*	1.07	8.3 ± 0.3^{d}*	1.38	7.36 ± 0.04^{b}*	1.23	8.0 ± 0.2^{c}*	1.34
B. animalis BB12	0	7.08±0.01	1.00	7.03±0.04	1.00	6.8±0.3	1.00	6.80±0.04	1.00
	6	7.05±0.02	1.00	7.31±0.01*	1.04	7.23±0.05*	1.06	6.69±0.04	0.98
	10	7.00 ± 0.06^{ab}	0.99	7.68 ± 0.02^{b}*	1.09	7.5 ± 0.2^{b}*	1.10	6.72 ± 0.02^{a}	0.99
	24	5.9 ± 0.2^{ab}*	0.83	6.1 ± 0.1^{b}*	0.86	7.41 ± 0.09^{c}*	1.09	5.6 ± 0.1^{a}*	0.83

[1]Ratio = $(\text{Log } N_i)/(\text{Log } N_0)$; N = mean (cfu/mL) at time i=6, 10 or 24 hrs; N_0 = mean (cfu/mL) at time 0 hrs. Different letters in the same row (24h) for each probiotic strain and spreadable cream indicate significant differences ($p<0.05$) for the viable cells and * for significant differences ($p<0.05$) in comparison to values at 0h.

10.4. Conclusions

Dairy products have been widely explored by industry and by scientific researchers due to their health benefits and continuously increasing demand by consumers. Hence, a functional dairy spreadable dairy cream combining whey cheese and greek type yoghurt was successfully formulated and explored through the incorporation of the extracts of *O. pinnatifida* obtained with Viscozyme and of *Ph. nameko* obtained with Flavourzyme. Heating pre-treatment, homogenization and product formulation were studied and duly adapted. Microbial stability, intrinsic previously identified functional properties (prebiotic and anti-diabetic potential) and sensory description and acceptance were assessed to evaluate functional food potentiality of dairy matrix chosen. Independent of the extract added, microbial stability of the spreadable dairy cream revealed a short shelf-life capacity of 5 days. Prebiotic potential of extracts was validated in the dairy matrix for both *L. acidophilus* La-5 and *B. animalis* BB12; however upon submission to simulated gastrointestinal conditions for digestion, growth promotion of *B. animalis* BB12 was partially lost in the case of the *O. pinnatifida* Viscozyme extract. Consumer acceptance and degree of liking toward the developed products was also assessed and overall results were promising in particular for the spreadable dairy cream incorporated with the *Ph. nameko* Flavourzyme extract which presented scores similar to those of the control. Product appraisal to identify specific sensory attributes driving product

acceptability is indeed of vital importance and formulation improvement or incorporation technology of extracts needs to be pursued to guarantee a better approximation to consumer expectations.

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Chapter 11

Concluding remarks

In this thesis, the search, selection and characterization of edible seaweeds and mushrooms for the identification of functional ingredients was pursued. Six edible seaweed species harvested from the Portuguese West coast and 5 cultivated edible mushrooms demonstrated good potential for further processing or for direct food and nutraceutical applications given their very good nutrient profiles.

The main composition and nutritional features for the 6 edible seaweed collected species were:
- Red seaweeds species registered the highest protein content but the lowest fat and sugar content whereas green and brown seaweeds stood out for their highest fat and sugar content, respectively;
- Low fat content (0.6 to 3.6%) was found in all 6 seaweeds albeit with a specific FA profile rich in palmitic acid, araquidonic acid and EPA;
- Higher total phenolic content was observed in the green seaweed *C. tomentosum* followed by the brown seaweed *S. muticum* and the red seaweed *O. pinnatifida*;
- All 6 seaweeds were characterized by high levels of minerals and some of the selected seaweeds (*S. muticum*, *S. polyschides* and *C. tomentosum*) may be included in the human diet to help solve problems with mineral deficiency, in particular, Ca, K, Mg and Fe, contributing significantly to the daily requirements intake of several countries;
- *G. gracilis* and *O. pinnatifida* were found to be mostly agar producers whereas *G. turuturu* was associated *to* agaroid-carrageenan hybrid polysaccharides;
- In the brown seaweeds *S. muticum* and *S. polyschides,* alginates and fucoidans were the main representative polysaccharides;
- In the green seaweed *C. tomentosum,* (1→4)-β-D-mannans, sulphated and unsulphated galactose residues were evidenced.

The main composition and nutritional features for the 5 cultivated edible mushrooms were:
- High contents in proteins and polysaccharides associated with low content of fat characterized the three *Pleurotus* species as well as *Pholiota nameko and Hericium erinaceus;*
- Higher content in mono and PUFAs than saturated FA characterized the 5 mushrooms FA profile with high linoleic acid concentration (>30 $gFA/100g_{fat}$);
- Highest total phenolic content was observed for *P. citrinopileatus* var. *cornucopiae* with 1140 µg cathecol $equiv/g_{dry\ mushroom}$;
- The 5 mushroom species may be considered good sources of K, Mg, P, highlighting K (2627-3736 $mg/Kg_{dry\ mushroom}$) as the most predominant, and of Zn, Cu and Fe; some contributing over 15% of their recommended daily intakes;
- The presence of β-glucans, α-glucans and glucan-protein complexes are among the main representative polysaccharides in the 5 mushroom species.

The use of several extraction methods sustainable and food compatible were applied to 3 selected seaweeds (*S. muticum*, *O. pinnatifida* and *C. tomentosum*) and 1 mushroom species (*Ph. nameko*):

- Enzymatic-assisted extraction revealed to be a promising tool to obtain extracts with attractive biological properties for both seaweed and mushrooms species;
- In general, it is somewhat difficult to pinpoint the most efficient extraction method with the highest biological potential since it depends on several factors: i) species; ii) extraction mode considering the duality: extraction yield *vs* chemical/biological properties; and iii) the type of analysis made;
- Enzymatic *S. muticum* extracts presented interesting antioxidant and prebiotic potential;
- Enzymatic *O. pinnatifida* extracts presented interesting free-radical scavenging activity given its high sulphated sugar content;
- Enzymatic *C. tomentosum* extracts presented a promising α-glucosidase inhibitory activity and consequently attractive anti-diabetic potential;
- All *Ph. nameko* extracts, in particular enzymatic extracts, presented interesting prebiotic potential. Promising α-glucosidase inhibitory activity was observed in UAE and EAE with Cellulase;
- Response surface methodology applied for optimizing HHP assisted extraction of bioactive polysaccharides from *S. muticum* showed improved extractability (higher extraction yields providing extracts concentrated in polysaccharides) but with lower antioxidant potential and absence of antidiabetic activities;
- Enzyme-assisted extraction was revealed as one of the best options in terms of a food compatible extraction method with higher yield rates and through the selection of a specific enzyme some biological properties may be favoured over others properties.

Considering the previous results, the next stage in this integrated research was the chemical characterization of the bioactive components present in four selected extracts from seaweeds (*S. muticum* extract obtained with Alcalase and *O. pinnatifida* extract obtained with Viscozyme) and mushroom (*Ph. nameko* extracts obtained with Cellulase and with Flavourzyme) where target biological activities were confirmed or more promising:
- In general, the selected enzymatic extracts enabled the recovery of important compounds with nutritional or biological significance;
- Enzymatic aqueous extraction enabled an important concentration of almost all macro and micro elements in comparison to the dry foods (seaweeds or mushroom), and in some cases nutritional value (an extract containing at least 15% of the mineral RDI value) was much enhanced, in particular for K and P in the *S. muticum* enzymatic extract; for K, Mg, Zn and Mn in the *O. pinnatifida* extract and for Mg, K and P in both *Ph. nameko* extracts;
- Overall, higher contents of monosaccharides, uronic acids and glucosamine were observed in the *O. pinnatifida* extract obtained with Viscozyme in comparison to the *S. muticum* extract;
- The two seaweeds extracts revealed a variety of sugars in variable molar ratios whereas in the mushroom extracts qualitative and quantitative profiles contrasted with those of seaweeds; monosaccharides were the major constituents found at higher contents and no uronic acids were detected;

- The nitrogen moiety found in these extracts, expressed as free amino acids was not particularly expressive in the case of seaweeds extracts from both qualitative and quantitative standpoints yet higher in mushroom extracts; *Ph. nameko* extract obtained with Flavourzyme was characterized by a 1.7 higher total amount of amino acids in comparison to the Cellulase counterpart and half of the amount was composed of important essential amino acids.

The selected extracts with potential biological properties, following their chemical characterization need now to be confirmed and consolidated in terms of their biological potential through the evaluation of *in vitro* stability to be further explored within the functional food perspective:
- All tested extracts influenced gut microbiota composition yet were associated with distinct combinations of human gut microbiota over fermentation;
- The digested *Ph. nameko* extract obtained with Flavourzyme was found to specifically promote growth and metabolic functionality of *Bifidobacterium* spp. while *Lactobacillus* spp. remained relatively unaffected and growth of *Clostridium histolyticum* and of *members* of the *Clostridium coccoides–Eubacterium rectale* group was inhibited. This selective increase in bifidobacteria coupled to a consistent increase in total SCFA and lactic acid production suggest its potential prebiotic character.
- Gut microbiota were able to ferment seaweed extracts, in particular that of *O. pinnatifida* obtained with Viscozyme, reflected in an increase in SCFA. Increase in SCFA was not always correlated with an increase in bacterial populations for the seaweed extracts.
- Although the *O. pinnatifida* extract did not demonstrate a selectivity concerning prebiotic potential, it was found to have a moderate ACE inhibitory activity.

The last stage of this thesis was the development of functional foods by incorporating the two most promising and validated extracts (*O. pinnatifida* obtained with Viscozyme and *Ph. nameko* obtained with Flavourzyme) in food matrices (spreadable dairy cream) and assessment of their biological and technological potential:
- A functional dairy spreadable dairy cream combining whey cheese and greek type yoghurt was successfully formulated and explored through the incorporation of the extracts of *O. pinnatifida* obtained with Viscozyme and of *Ph. nameko* obtained with Flavourzyme;
- Independent of the extract added, microbial stability of the spreadable dairy cream revealed a short shelf-life capacity of 5 days;
- Prebiotic potential of extracts was validated in the dairy matrix for both *L. acidophilus* La-5 and *B. animalis* BB12; however upon submission to simulated gastrointestinal conditions for digestion, growth promotion of *B. animalis* BB12 was partially lost in the case of the *O. pinnatifida* Viscozyme extract;
- Consumer acceptance and degree of liking toward the developed products was also assessed and overall results were promising in particular for the spreadable dairy cream incorporated with the *Ph. nameko* Flavourzyme extract which presented scores similar to those of the control.

As seen in chapter 10, the development of new functional dairy products is very challenging and it has to complete the consumer's expectations for palatable and healthy products as well as meet with regulatory demands. This dissertation responds to the latter requirement via validation of biological properties of the *Ph. nameko* Flavourzyme extract via both pre and post-digestion processes and administered either as nutraceutical or functional food (spreadable dairy cream). However, despite the important biological properties found for the *O. pinnatifida* Viscozyme extract, further optimisation may be performed both in terms of sensory acceptance in food as well as validation of biological properties particularly in post-digestion process. Hence, microencapsulation technology may be explored for this extract not only to protect it against undesirable degradation of compounds consequence of processing and/or physiological digestion, but also to contribute to the masking effect required to eliminate the unpleasant flavour and taste pointed out by some of the sensory panel members.

Additionally, alternative food matrices may be tested for incorporation of the selected extracts, either of dairy origin such as ice cream or cheese, or beverages or even dry cereal-based products. All procedures developed and studied in this dissertation will be required for testing of new functional food vectors.

To complement the integrated strategy of functional food development, besides widening food applications there is also a need for studies investigating the identified biological properties *in vivo* in clinical trials, as required for health claim applications. For example, the prebiotic effect of *Ph. nameko* extracts needs to be demonstrated in high quality, well-controlled human intervention studies and dose-effect relationships will need to be established and matched with technological potential of extracts and incorporation in different matrices. During these trials it is mandatory to control the diets, in order to detect their short-term effects. If a long-term effect is pursued then it will be important to assess the habitual food intake via food frequency questionnaires and 24-hour dietary recalls. In addition, fully identification and characterization of the polysaccharides present in the extracts and responsible for the biological properties would complete the chemical information of the most promising extracts.

Appendix I

Sensorial Analysis Questionnaire

mousse requeijão

Q27 Muito obrigada por participar nesta sessão de avaliação sensorial de MOUSSES DE REQUEIJÃO com propriedades funcionais. Ser-lhe-á pedido que prove 3 MOUSSES, uma de cada vez, por uma ordem predeterminada, e que responda a algumas questões. Leia cuidadosamente as instruções à medida que forem aparecendo no ecrã do seu computador. Por favor não troque impressões com os outros provadores. Não existem respostas certas ou erradas, o importante é podermos obter a sua opinião pessoal. Note que, uma vez respondidas as questões, o software não lhe permitirá retornar aos ecrãs precedentes. Não hesite em solicitar ajuda se tiver alguma dúvida ou se precisar de qualquer tipo de assistência. Se tiver alergias alimentares por favor não participe nesta avaliação sensorial.

Obrigada !

Q5 POR FAVOR INDIQUE Sexo:

- Feminino (1)
- Masculino (2)

Q7 Ano de nascimento:

- 1935 (1)
- 1936 (2)
- 1937 (3)
- 1938 (4)
- 1939 (5)
- 1940 (6)
- 1941 (7)
- 1942 (8)
- 1943 (9)
- 1944 (10)
- 1945 (11)
- 1946 (12)
- 1947 (13)
- 1948 (14)
- 1949 (15)
- 1950 (16)
- 1951 (17)
- 1952 (18)
- 1953 (19)
- 1954 (20)
- 1955 (21)
- 1956 (22)
- 1957 (23)
- 1958 (24)
- 1959 (25)
- 1960 (26)
- 1961 (27)
- 1962 (28)
- 1963 (29)
- 1964 (30)
- 1965 (31)
- 1966 (32)
- 1967 (33)
- 1968 (34)
- 1969 (35)
- 1970 (36)
- 1971 (37)
- 1972 (38)
- 1973 (39)
- 1974 (40)
- 1975 (41)
- 1976 (42)
- 1977 (43)
- 1978 (44)
- 1979 (45)
- 1980 (46)

- ❍ 1981 (47)
- ❍ 1982 (48)
- ❍ 1983 (49)
- ❍ 1984 (50)
- ❍

- ❍ 1985 (51)
- ❍ 1986 (52)
- ❍ 1987 (53)
- ❍ 1988 (54)
- ❍ 1989 (55)
- ❍ 1990 (56)
- ❍ 1991 (57)
- ❍ 1992 (58)
- ❍ 1993 (59)
- ❍ 1994 (60)
- ❍ 1995 (61)
- ❍ 1996 (62)
- ❍ 1997 (63)
- ❍ 1998 (64)
- ❍ 1999 (65)
- ❍ 2000 (66)

Q15 Nacionalidade:
- ❍ Portuguesa (1)
- ❍ Outra, por favor especifique: (2) _____

Q12 País de residência
- ❍ Portugal (1)
- ❍ Outra, por favor especifique: (2) _____

Q7 Nível de estudos
- ❍ 1º Ciclo do Ensino Básico (4º Ano) (3)
- ❍ 2º Ciclo do Ensino Básico (6º Ano) (2)
- ❍ 3º Ciclo do Ensino Básico (9º Ano) (4)
- ❍ 11º Ano ou 12º Ano (7)
- ❍ Curso Tecnológico/Profissional/Outros (Nível III) (5)
- ❍ Bacharelato (9)
- ❍ Licenciatura ou Mestrado (6)
- ❍ Doutoramento (8)

Q61 Em geral, quanto gosta dos produtos abaixo indicados?
_____ Requeijão (2)
_____ Iogurte (3)
_____ Queijo fresco (5)
_____ Cogumelos (6)

Q17 Costuma consumir requeijão?
- Sim, algumas vezes por semana (2)
- Sim, algumas vezes por mês (3)
- Sim algumas vezes por ano (4)
- Muito raramente (5)
- Nunca (6)

Q23 Por favor prove agora a mousse de requeijão 4P8.

Q25 GLOBALMENTE, QUANTO GOSTOU de 4P8?
- Gostei extremamente (1)
- Gostei muito (2)
- Gostei moderadamente (3)
- Gostei ligeiramente (4)
- Não gostei nem desgostei (5)
- Desgostei ligeiramente (6)
- Desgostei moderadamente (7)
- Desgostei muito (8)
- Desgostei extremamente (9)

Q26 Avalie agora pf cada um dos atributos abaixo da mousse 4P8:
_____ Aspeto (1)
_____ Odor (2)
_____ Sabor (3)
_____ Cremosidade (4)
_____ Consistência (5)

Q30 CONSUMIRIA a mousse 4P8, se ela estivesse disponivel no mercado a um preço conveniente?
- 10- Sim (1)
- 9- É quase certo (2)
- 8- Muito provavelmente (3)
- 7- Provavelmente (4)
- 6- Boa possibilidade (5)
- 5- Possibilidade média (6)
- 4- Possibilidade razoável (7)
- 3- Alguma possibilidade (8)
- 2- Possibilidade ligeira (9)
- 1- Possibilidade muito ligeira (10)
- 0- Não (11)

Q62 Comentários

Q161 Por favor prove agora a mousse de requeijão com cogumelos 6S1.

Q162 GLOBALMENTE, QUANTO GOSTOU de 6S1?

- ❍ Gostei extremamente (1)
- ❍ Gostei muito (2)
- ❍ Gostei moderadamente (3)
- ❍ Gostei ligeiramente (4)
- ❍ Não gostei nem desgostei (5)
- ❍ Desgostei ligeiramente (6)
- ❍ Desgostei moderadamente (7)
- ❍ Desgostei muito (8)
- ❍ Desgostei extremamente (9)

Q163 Avalie agora pf cada um dos atributos abaixo da mousse 6S1:

_____ Aspeto (1)
_____ Odor (2)
_____ Sabor (3)
_____ Cremosidade (4)
_____ Consistência (5)

Q164 CONSUMIRIA esta mousse, se ela estivesse disponivel no mercado a um preço conveniente?

- ❍ 10- Sim (1)
- ❍ 9- É quase certo (2)
- ❍ 8- Muito provavelmente (3)
- ❍ 7- Provavelmente (4)
- ❍ 6- Boa possibilidade (5)
- ❍ 5- Possibilidade média (6)
- ❍ 4- Possibilidade razoável (7)
- ❍ 3- Alguma possibilidade (8)
- ❍ 2- Possibilidade ligeira (9)
- ❍ 1- Possibilidade muito ligeira (10)
- ❍ 0- Não (11)

Q165 Comentários

Q166 Por favor prove agora a mousse de requeijão com extractos 7Q9.

Q167 GLOBALMENTE, QUANTO GOSTOU de 7Q9?

- ❍ Gostei extremamente (1)
- ❍ Gostei muito (2)
- ❍ Gostei moderadamente (3)
- ❍ Gostei ligeiramente (4)
- ❍ Não gostei nem desgostei (5)
- ❍ Desgostei ligeiramente (6)
- ❍ Desgostei moderadamente (7)
- ❍ Desgostei muito (8)
- ❍ Desgostei extremamente (9)

Q168 Avalie agora pf cada um dos atributos abaixo da mousse 7Q9:
_____ Aspeto (1)
_____ Odor (2)
_____ Sabor (3)
_____ Cremosidade (4)
_____ Consistência (5)

Q169 CONSUMIRIA esta mousse, se ela estivesse disponivel no mercado a um preço conveniente?

- 10- Sim (1)
- 9- É quase certo (2)
- 8- Muito provavelmente (3)
- 7- Provavelmente (4)
- 6- Boa possibilidade (5)
- 5- Possibilidade média (6)
- 4- Possibilidade razoável (7)
- 3- Alguma possibilidade (8)
- 2- Possibilidade ligeira (9)
- 1- Possibilidade muito ligeira (10)
- 0- Não (11)

Q170 Comentários

Q27 Muito obrigada pela sua participação